Innate immune responses induced by *Chlamydia pneumoniae* infection

Thesis

Innate Immune Responses induced by *Chlamydia pneumoniae* infection

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By

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Declaration

I declare that all the work in this thesis has been composed and performed by myself. Contributions to the work of this thesis by colleagues are fully acknowledged in the text.

This work has not been, and is not currently being submitted for candidature for any other degree.

Table of Contents

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
Ad-	Adenoviral
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CBA	Cytometric Bead Array
CD	Cluster of Differentiation
cHSP	chlamydial Heat Shock Protein
COPD	Chronic Obstructive Pulmonary Disease
CPAF	Chlamydial Protease Activity Factor
CRP	Cysteine Rich Protein
DAB	Diaminobenzidine
DALY	Disability Adjusted Life Years
DC	Dendritic Cells
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
EB	Elementary Body
Elafin	Elastase-Specific Inhibitor
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular-signal Regulated Kinase
Euo	early upstream open reading frame
FACS	Fluorescence Activated Cell Sorter
FAK	Focal Adhesion Kinase
FAM	6-carboxyfluorescein
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G-CSF	Granulocyte Colony Stimulating Factor
GLXA	Exoglycolipid
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
H_2O_2	Hydrogen Peroxide
HDC	Histidine Decarboxylase
HLA	Human Leukocyte Antigen

HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
ICAM	Intracellular Adhesion Molecule
IDO	Indoleamine-2, 3-dioxygenase
IFN	Interferon
IFU	Inclusion Forming Units
IgG	Immunoglobulin G
IL-	Interleukin
IMDM	Iscove's Modified Dulbeco's Medium
Inc	Inclusion protein
iNOS	inducible Nitric Oxide Synthase
IP	Immunoprecipitation
Jak	Janus Kinase
kDa	Kilo Dalton
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
Mbp	Mega base pairs
MCP	Macrophage Chemoattractant Protein
MdM	Monocyte derived Macrophage
MEK	Mitogen-Activated Protein Kinase Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MOI	Multiplicity Of Infection
MOMP	Major Outer Membrane Protein
MRI	Moredun Research Institute
mRNA	messenger RNA
NFκB	Nuclear Factor kappa B
NK	Natural Killer
NKR	Natural Killer Receptor
NO	Nitric Oxide
OMP	Outer Membrane Protein
p.i.	post infection
PIAS	Protein inhibitors of activated Stats
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEG	Polyethylene Glycol
PFU	Particle Forming Units
PGE_2	Prostaglandin E_2
PI	Propidium Iodide
PI 3-K	Phosphatidylinositol 3-Kinase
Pias	Protein Inhibitor of Activated Stats
POMP	Polymorphic Outer Membrane Protein
RB	Reticulate Body
REML	Restricted Maximum Likelihood
RNA	Ribonucleic acid
RNAi	RNA interference
RPE	R-Phycoerythrin
rRNA	ribosomal RNA
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	Svedberg
SDS PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLPI	Secretory Leukocyte Proteinase Inhibitor
SOCS	Suppressors Of Cytokine Signalling
SSC	Side Scatter
Stat	Signal Transducer and Activator of Transcription
TAMRA	Tetramethylrhodamine
Th	T helper
TLR	Toll-like receptors
TNF	Tumour Necrosis Factor
Trp	tryptophan
TTS System	Type III Secretory System
Tween 20	Polyoxyethylenesorbiton monolaurate
VCAM	Vascular Cell Adhesion Molecule
WCL	Whole Cell Lysate
α_1-PI	alpha-1 protein inhibitor

List of Figures

Abstract

In the current project innate immune responses to *C. pneumoniae* infection were studied, using a human *in vitro* model: a combination of lung epithelial cell lines (HEp-2 and A549) and primary blood-derived monocyte-derived macrophages (MdMs). The experiments were designed with a view to identify the host-pathogen interactions early in the infection that may lead to persistence and the development of chronic diseases such as atherosclerosis.

Infected epithelial cells and MdMs released IL-8 and IL-6, while MdMs also released IL-1β, TNF-α and IL-10, but not IL-12. The release of cytokines by infected cells was modulated by IL-17 treatment.

Infected MdMs, but not infected epithelial cells displayed an altered surface phenotype: CD14, CD40 and CD54 were up-regulated, whereas MHC class I and II, and CD45 were down-regulated. Medium derived from infected epithelial cells modulated surface profile of MdMs in a different manner: MHC class I and II, and CD91 were up-regulated, while CD14 and CD45 were down-regulated.

IFN-γ plays a key role in controliing chlamydial growth through induction of indoleamine 2,3 –dioxygenase (IDO). A549 cells were found not to control chlamydial growth although they inducibly expressed IDO mRNA. The expression of Suppressor of Cytokine Signalling 3 (SOCS3) was investigated and A549 cells constitutively expressed both SOCS3 mRNA and protein, while HEp-2 cells expressed only SOCS3 mRNA. SOCS3 expression was further up-regulated by infection in both cells. IFN-γ treatment of A549 cells did not up-regulate SOCS3 mRNA expression, but did so in HEp-2 cells. The intracellular mechanisms of inhibition of IFN-γ-mediated chlamydial control in these celis remained unclear and require further study.

The findings presented in this thesis are discussed in relation to a hypothesis that early immune responses of infected lung cells play a crucial role in the outcome of the infection and that they contribute to the establishment of persistence.

CHAPTER 1

INTRODUCTION

Chlamydiae are common pathogens that cause a number of serious but preventable diseases in both humans and animals. *Chlamydia trachomatis* has been long recognised as the most common cause of sexually transmitted disease and trachoma, the most common cause of preventable blindness worldwide. More recently, in the last decade or so, *Chlamydia pneumoniae* has emerged as a major cause of human respiratory disease. There has been a lot of research into host responses to chlamydial infections and the development of immunopathology; but considerable gaps in our knowledge remain. The work described in this thesis aimed to analyse the early innate immune responses in the lung following *C. pneumoniae* infections with a view to better understanding host-pathogen interactions.

In this chapter I give an overview of the current knowledge about Chlamydiae and *Chlamydia pneumoniae,* in particular. The rationale underpinning the experimental approach and the aims of the project are outlined at the end of the chapter.

1.1 Chlamydia pneumoniae *and disease*

1.1.1 Global burden of Respiratory Disease

According to the WHO (World Health Organisation), lower respiratory tract infections are the leading cause of disability adjusted life years (DALYs) and the third most common cause of death worldwide. In 1990, 4.3 million people died as a result of lower respiratory tract infections, accounting for 8.5% of the total number of deaths in that year. Almost all of these deaths occurred in developing countries (3.9 million). The projections for the next 20 years are that there will be a decrease in the importance of acute infections, but chronic infections are likely to increase both as a cause of death and of DALYs (Murray and Lopez, 1997c, Murray and Lopez, 1997d, Murray and Lopez, 1997b, Murray and Lopez, 1997a).

1.1.2 Diseases related to *Chlamydia pneumoniae*

Chlamydia pneumoniae is a Gram-negative, obligate intracellular pathogen that is recognised as a major cause of human respiratory disease. It is estimated that up to 50% of the population worldwide is infected by their early twenties. The *C. pneumoniae* seroprevalence is negligible in children under the age of five, except in developing and tropical countries. Seroprevalence increases rapidly at ages 5 to 14, reaches 50% at the age of 20 and continues to rise slowly to 70% to 80% at ages 60 to 70. There does not seem to be a significant difference in the seroprevalence between the sexes under the age of 15, but it is considerably higher among adult men than among adult women. It is not clear why this is so (Blasi et al., 1998, Kuo et al., 1995).

C. pneumoniae causes both acute and chronic respiratory infections and has been associated with a number of diseases; it is associated with 20-30% cases of chronic obstructive pulmonary disease (COPD), up to 10% of community acquired pneumonia cases and around 5% of pharyngitis, bronchitis and sinusitis (Soler et al., 1998, Gerard et al., 2000, Kuo et al., 1995). The expanding spectrum of disease sequelae associated with *C. pneumoniae* infection includes atherosclerosis and ischaemic heart disease, multiple sclerosis and reactive arthritis (Smieja et al., 2002, Sriram et al., 1999, Stratton and Sriram, 2003, Braun et al., 1994). There are also reported associations with cases of otitis media, pulmonary exacerbations of cystic fibrosis, development of non-atopic asthma and other clinical syndromes like erythema nodosum, sarcoidosis and meningoencephalitis (Block et al., 1997, Emre et al., 1996, Blasi et al., 2000, Erntell et al., 1989, Gaede et al., 2002, Anton et al., 2000)

1.2 *Classification of Chlamydiae*

Chlamydiae are members of the order Chlamydiales. In 1980 the order Chlamydiales had one family, the *Chlamydiaceae* that had just two species; *Chlamydia trachomatis* strains that were identified by their accumulation of glycogen in inclusions and their sensitivity to sulfadiazine, and *Chlamydia psittaci* strains that did not accumulate glycogen and were usually resistant to sulphadiazine (Skerman et al., 1980).

The development of DNA-based classification methods during the 1980s provided new techniques for differentiating chlamydial groups. DNA-DNA reassociation data supported eight chlamydial groups on the level of genus or species. This has led to the creation of two additional species, *Chlamydia pneumoniae* (Grayston et al., 1989a) and *Chlamydia pecorum* (Fukushi and Hirai, 1992). Taking this into consideration, a new classification was proposed with one family, the *Chlamydiaceae* containing one genus and four species (reviewed in (Herring, 1993). However, in 1993 a new isolate was identified forming a ninth chlamydial group of *C. trachomatis*-like-*Chlamydia* in swine (Kaltenboeck et al., 1993). The existence of nine clusters has further been supported by analyses of phenotype, antigenicity, associated disease, host range, biological data, genomic nuclease restriction and genomic data including phylogenetic analyses using the ribosomal operon (Everett and Andersen, 1997).

Species within the *Chlamydiaceae* family have 16S rRNA gene sequences that are more than 90% identical. However, four additional groups of Chlamydia-like organisms were identified with a more than 80% 16S rRNA sequence identity. These bacteria meet the requirement to be included into the Chlamydiales order since they are obligate intracellular pathogens and have a Chlamydia-like developmental cycle.

Taking the variety of Chlamydiae and the new Chlamydia-like organisms into account Everett *et al* proposed a reclassification of the order Chlamydiales. They created a new phylogenetic tree based on analysis of 16S rRNA sequence similarity clusters, analysis of full-length 16S and 23S rRNAs, and on phenotypic and ecological differentiation of these organisms (Everett et al., 1999). The proposed reclassification of the order Chlamydiales is represented in Table 1.1.

Table 1.1 The proposed reclassification of the order Chlamydiales. Table adapted from Everett et al (1999).

TAXON	TYPE/REFERENCE STRAIN
FAMILY I: *Chlamydiaceae*	
GENUS *Chlamydia*	
Chlamydia muridarum sp. nov.	**MoPn**
Chlamydia suis sp. nov.	**S45**
Chlamydia trachomatis	**A/Har-13**
Biovar trachoma	**C/PK-2**
Biovar LGV	**L2/434/BU**
GENUS *Chlamydophiia*	
Chlamydophila abortus sp. nov.	**B577**
Chlamydophila caviae sp. nov.	**GPIC**
Chlamydophila felis sp. nov.	**FP Barker**
Chlamydophila pecorum comb. nov.	**E58**
Chlamydophila pneumoniae comb. nov.	**TW-183^T**
Biovar TWAR	**TW-183**
Biovar Koala	**LPCon**
Biovar Equine	**N16**
Chlamydophila psittaci comb. nov.	**6BC**
FAMILY II:*Simkaniaceae* fam. nov.	
Simkania negevensis sp. nov.	**Z**
FAMILY III: *Parachiamydiaceae* fam. nov.	
Parachlamydia achantoamoebae sp. nov.	**Bn$_9$**
FAMILY IV:	**WSU 86-1044**

The revision includes three new families within the order of Chlamydiales. They were included on the basis of their developmental cycle and more than 80% 16S and/or 23S gene sequence identity. The new families include three new species (Table 1.1). The *Chlamydiaceae* family has been divided into two genera including five new species. The genus *Chlamydia* now consists of three species; *Chlamydia trachomatis; Chlamydia muridarum* and *Chlamydia suis*. The new genus *Chlamydophila* has been introduced with six species. *Chlamydophila abortus*, *Chlamydophila caviae* and *Chlamydophila felis*, all previously considered strains of *Chlamydia psittaci* have been established as separate species. The other members of the genus are *Chlamydophila pecorum, Chlamydophila pneumoniae* and *Chlamydophila psittaci*.

Members of the *Chlamydia* genus have 16S and 23S gene sequences that are ≥97% identical. The genome size is ~1 Mbp and there are two identical ribosomal operons.

Chlamydia spp produce glycogen to varying degrees, some have an extrachromosomal plasmid, and they show a variable sensitivity to suplhadiazine. The type species for the genus is *Chlamydia trachomatis*.

The complete 16S rRNA and 23S rRNA genes of *Chlamydophila* species are ≥95% identical. The genome is approximately 1.2 Mbp and contains a single ribosomal operon. The *Chlamydophila* species do not produce detectable quantities of glycogen, have varying morphology and varying resistance to sulfadiazine. Many *Chlamydophila* species contain an extrachromosomal plasmid (Everett et al., 1999). The type species for the genus is *Chlamydophila psittaci.*

Chlamydophila pneumoniae is considered to be primarily a human respiratory pathogen. The first isolate was obtained from the conjunctiva of a child in Taiwan in 1965 and was named TW183. Since then many different isolates have been obtained from humans worldwide. There have also been a number of isolates from animals (koala, horse, frog) (Storey et al., 1993, Girjes et al., 1994, Berger et al., 1999). DNA sequence analysis of the *ompA* gene (see section 1.3.1) indicates that the amino acid sequence of MOMP (Major Outer Membrane Protein) varies less than 6% among these isolates, and in other species the variation is greater than 30%. Recently the full genomes of two chlamydial isolates AR39 and CWL02, were sequenced and compared and found to be 99.9% identical. High homology between isolates worldwide seems to be characteristic for the species (Read et al., 2000, Shirai et al., 2000).

The proposed reclassification of Chlamydiales has caused an ongoing debate in the *Chlamydia* research community since its publication. There were calls for rejecting the new classification altogether; however the most recent suggestion is to merge the *Chlamydia* and *Chlamydophila* genera into one genus but keep 9 separate species of *Chlamydia*. The lack of acceptance of the new nomenclature is particularly true of *Chlamydia/Chlamydophila pneumoniae.* To demonstrate this, in the period between 2000 and 2004 there have been 1403 publications on *Chlamydia pneumoniae* and only 86 on *Chlamydophila pneumoniae* (search conducted on PubMed on 10th September 2004, limiting the search to title and period). In the light of this, the old name *Chlamydia pneumoniae* will be used throughout this thesis.

1.3 *Chlamydial structure*

The defining biological characteristic of the genus *Chlamydia* is its unique intracellular developmental cycle, characterised by two distinct forms: the small, infectious elementary body (EB) and the larger, dividing reticulate body (RB). The EB has a diameter of ~400 nm, with a dense structure and a rigid cell wall, and it is metabolically inactive. The RB is variable in diameter (500-1500 nm) and volume (10-100 times bigger that that of the EB); it is metabolically active and has an osmotically and mechanically fragile cell wall (Chi et al., 1987). There are also intermediate forms identified as part of the growth phase in the chlamydial cycle.

1.3.1 Membrane-associated structures

The chlamydial cell wall is composed of a number of different molecules including peptidoglycan, which has a minor function in the osmotic stability of Chlamydiae, and a number of different proteins. The protein present in the largest quantity is the MOMP. It is expressed in both EBs, where it is highly cross linked by disulphide bonds, and in RBs, where the cysteine residues are completely reduced and there are no disulphide bonds. This difference in the cross linking of MOMP causes different cell wall rigidity between RBs and EBs. MOMP functions as a porin that allows the passage of ATP, and it is functional only in the reduced form, i.e. in RBs (Wyllie et al., 1998). This suggests that the tightly packed, highly cross-linked MOMP structure found in EBs ensures their protection from the extracellular environment. Also, MOMP is the major antigenic protein in all chlamydiae except for *C. pneumoniae* (Campbell et al., 1990, Christiansen et al., 1997), where it may be masked by polymorphic outer membrane proteins (POMPs) (Christiansen et al., 1998).

POMPs are a group of surface proteins of high molecular weight and unknown function. There are 9 POMP genes in *C. trachomatis* and as many as 21 POMP genes in *C. pneumoniae* (Stephens et al., 1998). They are expressed by both EBs and RBs of *C. pneumoniae* and *C. abortus* (Knudsen et al., 1999, Longbottom et al., 1998).

The first proteins to be discovered in the EBs were the cysteine rich proteins OmcA and OmcB. They are not expressed in RBs (Hatch et al., 1984, Hatch et al., 1986).

OmcA is expressed on the surface while OmcB is thought to be located mostly in the periplasm, but it may be associated with the inner leaflet of the outer membrane (Everett and Hatch, 1995). The ratio of MOMPs to OmcA and OmcB on the surface of the EBs is 5 MOMP: 2 OmcA: 1 OmcB (Everett and Hatch, 1991). Other components of the chlamydial structure include the outer membrane proteins (OMPs), first discovered in *C. pneumoniae* (Perez et al., 1994) and other potential candidates for this group of proteins have been identified in other Chlamydiae (Stephens et al., 1998).

Raulston *et al* have shown that a portion of heat shock protein 70 (HSP70) is expressed on the surface as a part of the outer membrane complexes of chlamydial EBs. Also, under reducing conditions, HSP70 is exposed on the surface suggesting that it may have a role in chlamydial entry (Raulston et al., 1993, Raulston et al., 1998) Lipopolysaccharide (LPS) is expressed in all species of Chlamydiae and the predominant form of LPS found in cell culture resembles that of *Salmonella minnesota* Re mutant. It has a low endotoxic activity and this seems to be due to its structure (Kosma, 1999); however the biological function of LPS still needs to be elucidated. Other non-protein molecules present at the surface are a heparan sulphate-like glycosaminoglycan (GAG) and exoglycolipid (GLXA); however their function is not clear as yet.

1.3.2 The Nucleoid

The structure of the nucleoid seems to be unique to Chlamydiae; it has a variable density, being much denser in EBs than in dividing RBs; however its complete structure is not known. The nucleoid contains two histone-like proteins Hc1 and Hc2 encoded by *hctA* and *hctB* respectively. Other putative nucleoid proteins have been identified from the genome sequence including SET (CT737), SWIB (CT460) and Swi/Snf2 (CT555, CT708) (Stephens et al., 1998). The histone-like proteins are associated with DNA; Hc1 preferentially binds to supercolled DNA, whereas Hc2 binds equally well to linear and supercoiled DNA *in vitro*. This suggests that Hc1 may be largely responsible for the DNA compaction seen in EBs, whereas Hc2 may play a

subtle role in nucleoid morphology and perhaps a role in stage-specific gene expression (Pedersen et al., 1996a, Pedersen et al., 1996b).

1.4 *Chlamydial developmental cycle*

The chlamydial cycle was first described by light microscopy in 1932 by Bedson and Bland (Bedson and Bland, 1932) and it was further analysed in great detail by electron microscopy later. The cycle can be divided into four stages: attachment, entry, development and release. A schematic of the whole chlamydial developmental cycle is presented in Figure 1.1.

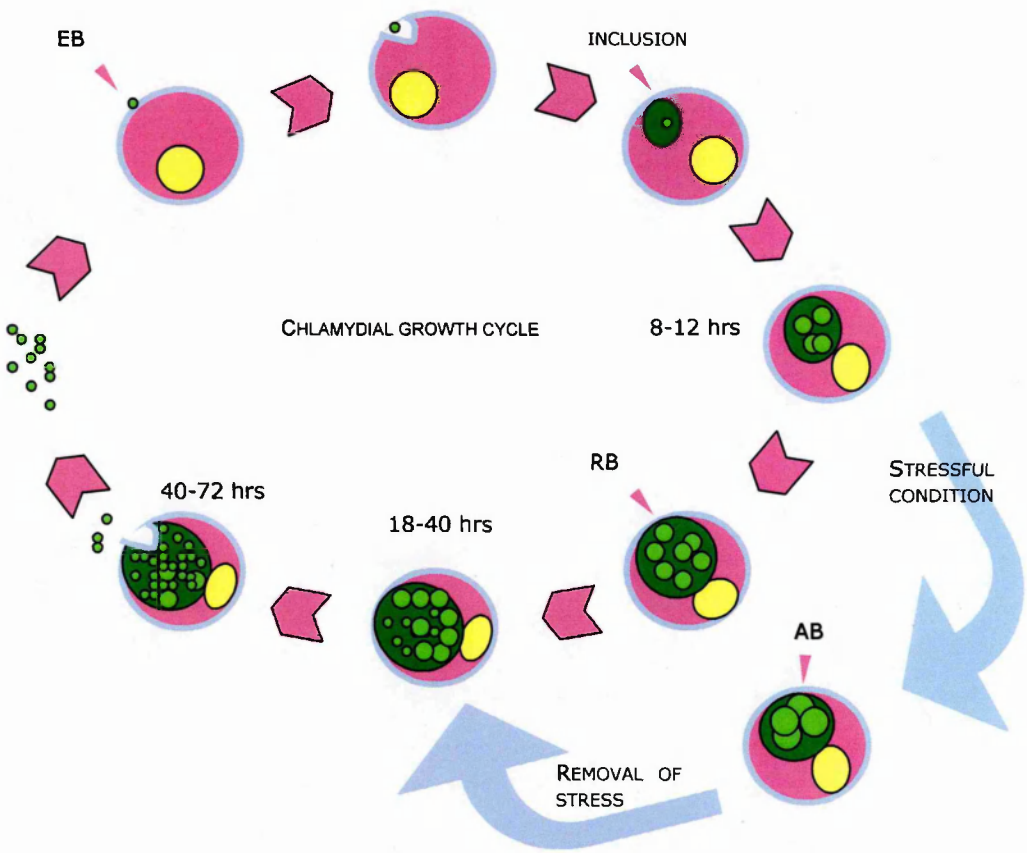


Figure 1.1 Chlamydial developmental cycle. **Key**: EB-elementary body; RB-reticulate body; AB-aberrant body.

Host cell-pathogen interactions during the attachment stage are not entirely clear and will be discussed further in section 1.4.1. After attachment, the EBs are internalised into the host cell and are found within a compact, membrane-bound vesicle called the inclusion (Figure 1.1). The inclusion does not fuse with the lysosomes, thereby

evading destruction, a process that is dependent on early protein synthesis by the organism. The reorganisation of the EBs into RBs begins within minutes after the internalisation, and after 8-12 hours almost pure RB populations are seen. The reorganisation is characterised by at least two events: dispersal of the condensed nucleoid structure to free the chromatin for increasing transcriptional activity, and loss of infectivity such that infectious particles are not recoverable upon destruction. RBs multiply by binary fission and are first observed dividing after about 12 hours.

In order to divide and grow, Chlamydiae need building blocks and these are provided by the host cell. The bacteria get their energy partly from the host cell by taking up ATP using ADP/ATP translocases Npt1Ct and Npt2Ct (Kalman et al., 1999). Npt1Ct mediates ATP transport by receiving ATP in exchange for ADP. Npt2Ct catalyses the net uptake of all four ribonucleoside triphosphates. However, Chlamydiae also express genes necessary for ATP generation (Hatch et al., 1982, Stephens et al., 1998). Analysis of *C. trachomatis* gene transcription in active versus persistent infection suggests that, in the first phase of active infection, ADP/ATP exchange provides the energy required for metabolism with bacterial glycolysis supplementing host-derived ATP, while in persistent infection the primary energy source is host cell-, and not chlamydia-produced ATP (Gerard et al., 2002).

The inclusion expands as the RBs multiply to accommodate the growing number of chlamydial particles. The conversion of RBs to EBs is not a synchronised process, it starts about 18 hours after the infection and by 30-40 hours there is a growing number of EBs present in the inclusion (Moulder, 1991). The position of the EBs within the inclusions varies between chlamydial species. In *C. trachomatis,* EBs accumulate in the lumen of the inclusion, and the dividing RBs are in proximity to the inclusion membrane, while in *C. pneumoniae* they mix together in the lumen (Wolf et al., 2000). The cell cycle is completed with the release of chlamydial particles from the host cell between 36 and 72 hours after the infection, depending on the species or strain of *Chlamydia*. This can happen either by the lysis of the host cell or by

expulsion of whole inclusions without cell death. The initial number of EBs influences the host cell fate. The infection can slow down the host cell DNA replication and ultimately lead to cell death. Infected cells can divide and give two infected daughter cells or one infected and one uninfected cell and the latter case may play a role in limiting the spread of infection and in establishing persistent infections (Moulder, 1991).

Persistence can be initiated by interruption of the "normal" developmental cycle by a number of different conditions and agents, including antibiotics, nutrient deprivation, and immune factors especially IFN-γ. In the presence of these factors RBs cease to divide and are transformed into large, morphologically aberrant forms called aberrant bodies (ABs). Specific markers for the persistent state have not been found, but chlamydial gene expression is altered in persistent infections as compared to active infections. Genes for DNA synthesis are expressed, but genes for cell division are not (Byrne et al., 2001, Mathews et al., 2001). If the stressful condition is removed the replicative cycle can continue and infectious EBs can be produced.

1.4.1 Attachment and Entry

The processes and molecules involved in the interactions between chlamydial particles and the host cell are still not clear, however a number of host cell molecules have been suggested as potential receptors for chlamydial particles. N-acetylneuraminic acid and N-acetylglucosamine have been implicated as components of a putative host cell receptor. However, it had been shown that *C. trachomatis* elementary bodies attach as efficiently to insect cells lacking these molecules as they do to the Chlamydiae-susceptible McCoy cell line thus leaving this hypothesis open to further investigation (Allan and Pearce, 1987).

Other molecules such as heparin and heparan sulphate have also been implied in the attachment process. When HeLa cells are treated with heparin and heparan sulphate or heparitinase, the infectivity of *C. trachomatis* L2 is consistently inhibited (Zhang and Stephens, 1992, Taraktchoglou et al., 2001). Also, exogenous GAGs were found

to inhibit the attachment of *C. pneumoniae* to human epithelial cells (Wuppermann et al., 2001). Binding of heparin to the organism and the enzymatic removal of heparan sulphate moieties from the host cell surface results in the marked decrease in the infectivity of *C. pneumoniae* in CHO cells (Wuppermann et al., 2001). Given that the presence or absence of different molecules can affect the attachment processes, it is likely that chlamydial attachment and entry is a dynamic process involving multiple receptors on the host cell.

Similar difficulties have been encountered in identifying molecules on the organism involved in chlamydial attachment to the host cells - chlamydial adhesin(s). The differential effect of trypsinization on the attachment of a serovar B, an LGV biovar strain of *C. trachomatis,* to cells has lead to the suggestion that chlamydial MOMP may be involved in the attachment process (Su et al., 1988). Also, MOMP was shown to bind to heparan sulphate on the host cell surface and this observation further supports its role as a chlamydial adhesin (Su et al., 1990, Su et al., 1996).

The attachment of *C. pneumoniae* is affected by its morphology. *C. pneumoniae* TWAR strain EBs have two distinct ultrastructural morphologies: the classic coccoid morphology of the *Chlamydiaceae* family (Popov et al., 1991) or the prototypical pear-shaped EB (Grayston et al., 1989b). The entry and ingestion of the pear-shaped EBs into the cells is different from the EBs of other Chlamydiae. They preferentially first attach to the host cell surface by the pointed end and then bind to other parts by means of cell wall protrusions. The host cell membrane invaginates and the *C. pneumoniae* EBs are internalised in vacuolated inclusions (Grayston et al., 1989b). Using scanning and transmission electron microscopy, Coombes & Mahony (2002) demonstrated that attachment of *C. pneumoniae* to host cells induces the appearance of microvilli on host cell membranes. Invasion occurs between 30 and 120 min after cell contact, with subsequent loss of membrane microvilli (Coombes and Mahony, 2002).

The invasion of HEp-2 cells by *C. pneumoniae* causes a change in the intracellular signalling pathways. There is a rapid increase in MEK-dependent phosphorylation and activation of ERK1/2, followed by PI3-kinase-dependent phosphorylation and activation of Akt. Tyrosine phosphorylation of focal adhesion kinase (FAK) precedes its appearance in a complex with the p85 subunit of PI3-kinase during chlamydial invasion. Isoform-specific tyrosine phosphorylation of the docking protein Shc also occurs at the time of attachment and entry. Chlamydial entry (but not attachment) can be abrogated with specific inhibitors of MEK, PI3-kinase and of actin polymerization. This suggests that activation of cell signalling pathways and an intact actin cytoskeleton are essential strategies for *C. pneumoniae* invasion of epithelial cells (Coombes and Mahony, 2002)

Vesicles containing endocytosed elementary bodies are delivered into close proximity with the host cell Golgi apparatus approximately two hours after infection. Translocation of the chlamydial entry vacuole requires the active participation of Chlamydiae, because If early chlamydial protein synthesis is blocked with the antibiotic chloramphenicol, the vacuole-bound elementary bodies remain distributed throughout the cell cytoplasm (Scidmore et al., 1996). Translocation of early *C. trachomatis* vacuoles to the peri-Golgi region probably involves the host cell cytoskeleton, as aggregated elementary bodies are associated with the micro-tubule organising centre of the cytoskeleton, while microtubule inhibitors block the aggregation of early inclusions (Clausen et al., 1997).

The chlamydial endosome lacks any of the classic host cell markers of either early endosomes (transferrin or transferrin receptor) or late endosomes (cation-independent mannose-6-phosphate receptor) and is essentially non fusogenic with host cell lysosomes. This probably relates to some intrinsic property of the elementary body cell wall, as normal endosome maturation is prevented if the endosome contains purified chlamydial cell walls or if chlamydial protein synthesis is blocked by antibiotics (Hackstadt, 1998). Blockade of normal endosomal maturation occurs as an early event

in the first two hours following infection, before the nascent vacuole starts to intercept exocytic host cell membrane transport from the Golgi apparatus. Subsequently, chlamydial gene products modify the vacuole so that it becomes fusogenic with a subset of sphingomyelin and cholesterol-containing exocytic vesicles from the Golgi apparatus (Carabeo et al., 2003). Glycero-phospholipids acquired by the chlamydial vacuole from the Golgi apparatus, the mitochondria and endoplasmic reticulum, are subsequently modified by the Chlamydiae. The result is that Chlamydiae have a phospholipid composition that is much closer to that of the host cell than to that of other bacteria, including the unusual presence of cholesterol (Wylie et al., 1997, Carabeo et al., 2003).

1.4.2 Development and gene expression

Since the genomes of *C. trachomatis* and *C. pneumoniae* have been sequenced, a whole new area of chlamydial biology has been opened up for investigation and extensive work has been done on analysing their gene expression profiles. Genes have been divided according to the time of their expression in the cycle into three groups: **early transcripts** - detected 2 to 6 hours after infection, during the germination of EBs to RBs; **mid-cycle transcripts** - appearing between 6 and 12 hours after infection during the growth and multiplication of RBs, and **late transcripts**, which appear between 12 and 20 h after infection, during the differentiation of RBs to EBs. Collectively, early gene functions appear weighted toward initiation of macromolecular synthesis and the establishment of the intracellular niche by modification of the inclusion membrane. Late gene functions appear to be predominantly those associated with the terminal differentiation of RBs back to EBs (Shaw et al., 2000). An overview of temporal gene expression along with references is presented in Table 1.2 below. This Table was adapted from www.chlamydiae.com with minor changes. Expression and regulation of inclusion proteins, Type III Secretory System and heat shock proteins will be discussed in more detail in the following sections.

Table 1.2 Molecular events during the chlamydial growth cycle. Abbreviations used: **EB** elementary body; **RB** reticulate body; **p.i.** post infection; **MOMP** major outer membrane protein; **CRP** cysteine rich proteins; **HSP** heat shock protein; **euo** early upstream open reading frame.

TIME P.I.	EVENT	COMMENT	REFERENCES
Zero	Endocytosis	Release of pre-formed proteins via TTS- system to prevent lysomal fusion?	
~15 mins	EB to RB	EB commences protein synthesis	(Wichlan and Hatch, 1993)
~60 mins	EB to RB	Peak transcription of ***euo*** gene. Undetectable at 8 hrs after infection. Breakdown of histone (Hc1). Selected transcriptional activation / repression of genes at AT rich sites? Transcription of heat shock proteins **GroEL** and **GroES** Transcription of **IncD, IncE, IncF, IncG, CT228, CT229, CT147**	(Belland et al., 2003, Plaunt and Hatch, 1988, Wichlan and Hatch, 1993, Kaul et al., 1997, Kaul and Wenman, 1998, Nicholson et al., 2003, Zhang et al., 1998, Zhang et al., 2000)
1-2 hrs	EB to RB	Reduction of disulphide-linked MOMP to monomer detectable. Not so for CRP. MOMP accumulates throughout cycle. **IncD** transcription continues.	(Hatch et al., 1986)
2-8 hrs	EB to RB	Transcription of metabolic and house keeping genes, some specific to early and mid cycle	(Zhang et al., 1998)
8-16+ hrs	RB division	Inc proteins; projections into inclusion membrane. **IncA** may partly mediate inclusion fusion in *C. trachomatis*. RB-specific proteins include p52, *C. trachomatis* TroA and p242. Lipids from exoglycolytic pathway intercepted for incorporation into the expanding chlamydial endosome.	(Rockey et al., 1995)
8-16+ hrs	RB division	RBs lack histone proteins DNA diffuse permitting gene transcription and translation.	
8-16+ hrs	RB division	RB lacks Omp2 and Omp3 CRPs, leading to fragile but permeable wall structure.	(Hatch et al., 1986, Everett and Hatch, 1995)
15 hrs +	RB to EB	Stimulated by environmental or contact sensing? Upregulation of histone proteins. DNA condensation on histone proteins HctA, HctB Upregulation and cross linking of Omp2 and Omp3, perhaps involving CT780, CT783 and adenovirus-related thiol proteases.	(Belland et al., 2003, Nicholson et al., 2003, Sardinia et al., 1988)
40 hrs +	Release	Productive cycle only. Release by lysis or exocytosis	

1.4.3 Regulation of gene expression

Chlamydial development involves the expression of many genes that need to be strictly regulated. Gene expression is at least in part regulated by sigma (σ) factors that modulate the binding of RNA polymerase to specific gene targets. An RNA polymerase core enzyme binds to a σ factor. The enzyme is then capable of recognising the promoter and it can initiate transcription at the transcription start site, known as the +1 site. Different σ factors allow the relevant RNA polymerase to initiate transcription from different promoter sequences. This provides a mechanism of gene regulation. If the σ factors themselves are differentially expressed, it follows that the genes under their control will be transcribed differentially. The genome sequence of *C. trachomatis* has revealed three σ factors (Stephens et al., 1998). They are σ^{66}, σ^{54} and σ^{28} and there are also several predicted enhancer binding proteins, such as NifS. Sigma factors are probably capable of recognising different chlamydial gene promoters as well as the -10 (TATAAT) and -35 (TTGCCA) boxes that are analogous to *E. coli* promoters recognized by E-σ^{70} (Tan et ai., 1998, Mathews and Sriprakash, 1994). Chlamydial σ^{66} promoters are unlike those of *E.coli* σ^{66} (Mathews and Stephens, 1999). A number of σ^{54} promoters have been identified and a number of genes regulated have been proposed; however σ factors and promoter recognition alone are unlikely to be sufficient for control of the chlamydial development cycle (Mathews and Timms, 2000).

1.4.3.1 Inclusion proteins

In order to ensure their survival within the host ceil, Chlamydiae manipulate the intracellular processes. It is thought that they do so through the inclusion membrane. In 1994 Rockey and Rosquist identified a 39 kDa protein expressed in the infected cells and RBs, but not EBs, of *Chlamydia psittaci* GPIC (Rockey and Rosquist, 1994). The protein was located in the inclusion membrane and was named inclusion protein A, IncA (Rockey et al., 1995). Subsequently it was shown that other Chlamydiae also express Inc proteins and the *inc* genes are conserved within the Chlamydiaceae family. At least 11 different Inc proteins have been identified in *C. trachomatis.* Using

computer modelling, 90 proteins in the *C. pneumoniae* J138, and 36 proteins in the *C. trachomatis* serovar D sequences were identified that have hydropathy profiles similar to the Inc proteins. Only a few Inc-like open reading frames were found in other organisms, suggesting that these proteins are exclusive to the Chlamydiae. Comparative genome analysis suggests that the Inc-like open reading frames have multiplied and diverged as orthologues (copied genes that retain the same function as the original) and paralogues (copied genes that have a different function than the original) in the chlamydial genomes, and that some of them lack the N-terminal portion or encode a split form (Bannantine et al., 2000, Toh et al., 2003).

The Inc-like proteins constitute a large family and they are likely to play an important role in chlamydial infections throughout the life cycle. *C. caviae* IncA was found to face the cytoplasmic side of the inclusion membrane, and is phosphorylated by host cell kinases at its serine and threonine residues (Rockey et al., 1997). Given the importance of kinases in the intracellular signalling machinery of the host cell, IncA might have a role in interfering with these pathways to serve the pathogen. Also certain *C. trachomatis* strains do not express an IncA homologue and produce uncharacteristic multiple inclusions in cells instead of one large inclusion indicating that IncA mediates the fusion of small homotypic inclusions containing chlamydial RBs into one large inclusion (Suchland et al., 2000). In addition Inc proteins may be involved in preventing fusion between the chlamydial endosome/inclusion with lysosomes, and in getting nutrients from the cytosol of the host cell across the inclusion membrane to the Chlamydiae (reviewed in Wyrick, 2000). As seen in Table 1.3, Inc proteins are expressed early in the development cycle. Even though they are membrane-associated proteins, they generally lack the characteristic signal peptides that target nascent proteins into bio-membranes. One speculation is that Chlamydiae inject the Inc proteins into inclusion membrane using a Type III Secretory System, discussed below.

1.4.3.2 Type III Secretory System

The Type III Secretory System (TTS system) functions as a 'molecular syringe', enabling Gram-negative bacteria to inject virulence-related proteins into the cytoplasm of host cells (Hueck, 1998). The TTS system differs from all other bacterial secretion mechanisms in that it requires, and is triggered by, the intimate contact of the bacterium with a host cell membrane. The TTS system is found in a wide range of Gram-negative bacteria including *Yersinia*, *Salmonella*, *Shigella*, *Escherichia*, *Pseudomonas*, *Bordetella*, *Burkholderia*, a number of plant pathogens or symbionts, and in *Chlamydia* (Winstanley and Hart, 2001).

TTS systems are known to consist of several elements, including the following: structural elements within the bacterial membrane, as well as those that are assembled upon contact with the host cell and that extend into the host cytoplasm (e.g., *ysc* genes); effector proteins which are released into the host cell to modulate host cell function (e.g. *yop* genes); a translocator apparatus that provides a pore within the host cell membrane for delivery of effectors (e.g. translocator genes *yopB*, *yopD*, and their chaperone *lcrH*); and specialized chaperones which function to stabilize and assure efficient secretion of translocator proteins, and also to regulate expression of some of the TTS genes (e.g., *syc* genes).

The genetic organisation of the TTS system in Chlamydiae does not form "pathogenicity islands"; rather, the genes are scattered over at least four different regions of the genome (Stephens et al., 1998). In order to confirm that *C. trachomatis* possesses a functional TTS system, the expression of genes encoding products with similarity to proteins from other TTS systems, has been analysed. These include CopN, a putative secreted protein homolog of the YopN TTS-secreted protein of *Yersinia*. CopN is expressed mid-cycle, and it is found in the inclusion membrane 20 hours after the infection. Also, when CopN is transfected into *Yersinia enterolytica*, it is expressed and secreted by the TTS-system, thereby confirming that Chlamydiae produce proteins that can be secreted via a TTS mechanism (Fields and Hackstadt, 2000). As

mentioned earlier there are indications that Inc proteins may be injected into the inclusion by a TTS-system; however Incs are expressed as early as two hours post infection, while TTS proteins are expressed mid cycle. Recently the expression of TTS-system was demonstrated on purified EB extracts by detecting a core apparatus protein CdsJ (Fields et al., 2003). This group also confirmed early activity of the TTS-system by secretion of IncC via the *Yersinia* TTS apparatus (Fields et al., 2003).

Type III secretory system might also have a role in chlamydial survival. In IFN-γ induced persistent infections, *C. pneumoniae* TTS protein ScTN is expressed along with MOMP, heat shock protein 60 and others, and this expression profile might help Chlamydiae to resist stress from effectors of the Th1-type immune response (Molestina et al., 2002).

Proteomic analysis of *C. pneumoniae* EBs identified 167 genes representing 15% of the coding capacity of the genome, including 31 hypothetical proteins (Vandahi et al., 2001). All of the data are available on the Aahrus database at www.gram.au.dk. A number of proteins of the putative *C. pneumoniae* type III secretion apparatus have been shown to be expressed. They are listed in Table 1.3.

Table 1.3 TTS system proteins of *C. pneumoniae*

Protein	Gene	Aarhus accession
T III secretion: low calcium response protein	*lcrE* or *Copn* or *sctw*	Q9Z8L4
T III secretion: YOP C/Gen Secretion Protein D	*yscC* or *sctC*	Q9Z7K3
T III secretion: YOP N (Flagellar Type ATPase,)	*yscN* or *sctN*	Q9Z7J8
T III secretion: YOP L translocation protein	*yscL or sctL*	Q9Z780

Another study analysed the temporal expression of TTS genes in *C. pneumoniae*. There are at least 13 genes that are homologous with other known TTS systems. The *groEL-1* (HSP60), *ompA*, and *omcB* genes were used as markers for the early, middle, and late stages of the developmental cycle. TTS genes were expressed as follows: early stage (1.5 to 8 h), *yscC, yscS, yscL, yscJ* and *lcrH-2*; middle stage (12 to 18 h), *lcrD, yscN, and yscR*; and late stage (24 h), *lcrE, sycE, lcrH-1* and *yscT*. IFN-γ

treatment affected some of the genes either by down-regulation or by changing the time of expression (Slepenkin et al., 2003).

1.4.3.3 Heat Shock Proteins

HSPs are a ubiquitous family of highly conserved proteins that stabilise cellular proteins during heat shock, infection and inflammation. Chlamydiae have seven heat shock-related chaperone proteins (groEL1, groEL2 groEL3, groES, grpE, dnaJ, dnaK). These proteins are up-regulated in response to heat or oxidative stress or to IFN-γ and other products of cell mediated immunity (Byrne et al., 2001). GroEL/HSP60 and GroES are expressed within one hour after infection (Belland et al., 2003). The σ factor that regulates heat shock protein expression has not been identified; however chlamydial HrcA has been demonstrated to be a regulator of chlamydial heat shock gene expression acting in conjunction with a cis-acting DNA element called CIRCE as a repressor-operator pair. HrcA repressed the *in vitro* transcription of a chlamydial heat shock promoter in a promoter specific manner (Wilson and Tan, 2002). The role of chlamydial heat shock proteins in immune responses will be discussed later in this chapter.

1.5 ***Immune responses to chlamydial infections***

Research to date has shown that the protective immunity to chlamydial infections is mediated primarily by a Th1-type response. Early cytokine production by the innate arm of the immune system is followed by an adaptive response dominated by CD4+ and CD8+ T cells. An overview of immune responses and the contribution of different cell types and cytokines are discussed in the following section.

1.5.1 Epithelial cells

Epithelial/mucosal surfaces are the primary target for *Chlamydia* spp. infections. Chlamydiae can infect the respiratory tract (*C. pneumoniae, C. muridarum, C. felis, C. suis*), urogenital tract (*C. trachomatis* LGV biovar), the conjunctiva (*C. trachomatis* trachoma biovar, *C. felis, C. suis, C. pecorum*), the gastrointestinal tract (*C. psittaci*) and also the trophoblast layer of the placenta (*C. abortus, C. pecorum*). Epithelial

surfaces are a physical barrier to the internal environment, but also have a pivotal role in the innate and early immune response to pathogen challenge.

The airway epithelium performs many homeostatic functions, such as gas transport, cellular repair and proliferation, barrier protection and ion and fluid transport. It can also regulate local inflammatory and immune responses by producing a range of cytokines (IL-6, IL-11) and chemokines (IL-8, MCP), as well as lipid (PGE_2) and peptide (endothelin, vasopressin) mediators and reactive oxygen species (NO, H_2O_2) (Polito and Proud, 1998). *C. pneumoniae* infection of airway epithelial cells modulates these responses. A number of host transcription factors are activated and this is followed by cytokine production. GR, C/EBP-β (CCAAT-enhancer binding protein beta) and NFκB (nuclear factor kappa B) are activated between 1 and 6 hours post-infection (Gencay et al., 2003). NFκB activation is followed by release of IL-8 and PGE_2 and up-regulation of ICAM-1 adhesion molecule. This is followed by subsequent transepithelial migration of neutrophils (Jahn et al., 2000) Another study has shown expression of IFN-γ, TNF-α and IL-8 mRNA in A549 cells in response to *C. pneumoniae* infection, but only IL-8 protein was found in the supernatants. Expression of IL-1β and IL-6 mRNA or protein was not affected (Yang et al., 2003). Infected lung epithelial cells *in vivo* are also induced to express histidine decarboxylase (HDC), an enzyme that produces histamine, and this is associated with the production of IL-4 in the lung, thereby supporting the association of *C. pneumoniae* infections with allergy and asthma (Burian et al., 2003).

1.5.2 Neutrophils

Neutrophils have an important role in the early immune response in chlamydial infections. There is evidence of early neutrophil infiltration (by day 2) into the lung, in a mouse model of *C. pneumoniae* pneumonitis (Yang et al., 1994) and *in vitro* data showed release of IL-8 by infected endothelial cells followed by increased trans-endothelial migration of neutrophils (Molestina et al., 1999). This implies that neutrophils might have a function of early IFN-γ production and T cell recruitment. *C. pneumoniae* can infect and multiply in the neutrophils themselves, and render them

resistant to apoptosis by autocrine production of IL-8, thereby providing another reservoir for spreading the infection (van Zandbergen et al., 2004).

Chlamydia-mediated recruitment of neutrophils might also be aided by IL-17. IL-17 is crucial in immune responses to *Klebsiella pneumoniae* infections, and when it is absent there is delayed infiltration of neutrophils, leading to higher bacterial loads and higher mortality in infected mice (Ye et al., 2001a, Ye et al., 2001b). IL-17 will be discussed in detail in chapter 4. Neutrophil recruitment in *C. pneumoniae* infections might support the association with chronic respiratory diseases such as COPD and chronic bronchitis (Sethi, 2000, Beaty et al., 1991, Blasi et al., 1993, Von Hertzen et al., 1996). Acute exacerbations of these are associated with an increased number of neutrophils in the airways (Selby et al., 1991). Clearance of *C. abortus* infection in mice is associated with a pronounced infiltration of neutrophils into the liver and early IFN-γ production (Del Rio et al., 2000). In a pregnant mouse model of *C. abortus* infection, neutrophils seem to be crucial for the recruitment of CD8+ T cells into the liver. In secondary infections, depletion of neutrophils results in lower levels of early IFN-γ and TNF-α production and higher bacterial loads (de Oca et al., 2000, Montes de Oca et al., 2000).

Neutrophils also play a role in host immunity to *C. trachomatis* infections. As with *C. abortus*, their depletion leads to higher bacterial loads in mice infected with *C. trachomatis* (Barteneva et al., 1996). Infected epithelial cells release IL-8 and this triggers migration of neutrophils to the infection site. The recruitment and activation of neutrophils differs between disseminating and non-disseminating serovars (Dessus-Babus et al., 2000, Dessus-Babus et al., 2002). *C. trachomatis*-recruited neutrophils are activated to release defensins. Neutrophil defensins appear to participate in the host defence in ascending pelvic infection and the pathogenesis of pelvic inflammatory disease. Defensins are small antimicrobial peptides, part of the innate immune responses, that show antimicrobial effects *in vitro* (Wiesenfeld et al., 2002). Natural antimicrobial peptides will be discussed in detail in chapter 7.

1.5.3 Natural killer (NK) cells

Natural killer cells are non-classical lymphocytes, cellular components of the innate immune system that do not express antigen-specific receptors at their surface. Their effector function consists of target cell lysis, as well as in Th1 cytokine and chemokine secretion. NK cytotoxicity appears not to be important for clearance of *C. pneumoniae* infection; however they might contribute by producing IFN-γ in the early stages of the infection, and this could in turn activate macrophages and T cells (Rottenberg et al., 2000). A similar effect is seen in genital *C. trachomatis* infection, where the NK cytotoxicity seems to be impaired but IFN-γ is important (Tseng and Rank, 1998, Mavoungou et al., 1999).

Natural killer cell activity is controlled by a concert of activating and inhibitory signals. The interaction of NKR (natural killer cell receptor) with MHC (Major Histocompatibility Complex) molecules inhibits NK cytotoxicity and cytokine production, while failure to express MHC renders cells susceptible to NK-mediated lysis (Moretta et al., 2000). *C. pneumoniae* infected monocytes produce IL-10 that down-regulates MHC class I production. This might reduce the presentation of bacterial epitopes by MHC and decrease the ability of CD8+ T ceils to eliminate infected cells (Caspar-Bauguil et ai., 2000). However, down-regulation of MHC might allow NK cells to recognise *C. pneumoniae* infected cells and thereby protect the host.

1.5.4 Monocyte-derived Macrophages

1.5.4.1 Early contribution of MdMs to anti-chlamydial immunity

Monocyte derived macrophages (MdMs) have a central role in chlamydial infections. Alveolar macrophages found in the lung within 2 days of infection *in vivo* in Swiss Webster mice (Yang et al., 1994). *Ex vivo* human alveolar macrophages are activated to produce IL-8, IL-1β, TNF-α and reactive oxygen species followlng infection. The infection also causes up-regulation of MHC class II, expression while there is no change in ICAM-1 expression (Redecke et al., 1998). Mouse bone marrow-derived macrophages infected *ex vivo* produce IFN-γ early after the infection, in an IFN-α/β-dependent, IL-12-independent manner, and this contributes to control of infection

(Rothfuchs et al., 2001). MdMs support *C. pneumoniae* infection *in vitro.* Infection can induce differentiation of monocytes into macrophages, cells are activated to produce TNF-α, IL-1β, IFN-γ and IL-6, and there is increased CD14 and ICAM-1 expression on the cell surface (Quinn and Gaydos, 1999, Kaukoranta-Tolvanen et al., 1996, Heinemann et al., 1996, Yamaguchi et al., 2002). In some cases *in vitro* growth of *C. pneumoniae* in monocyte/macrophages is restricted. The amount of infectious progeny released from infected MdMs *in vitro* varies and seems to depend on the length of time in culture and also on the level of differentiation of MdMs, but the number is always lower compared to epithelial cells (Quinn and Gaydos, 1999, Kaukoranta-Tolvanen et al., 1996). Even when the growth is restricted, residual chlamydial epitopes are enough to induce proliferation of T cells up to a week after chlamydial mRNA is detected (Airenne et al., 1999). IFN-γ treatment of infected MdMs does not affect the rate of chlamydial growth, but high doses of the cytokine diminish the number of infectious progeny released by the MdMs, suggesting that immunological pressure can cause persistence (Airenne et al., 2000).

1.5.4.2 Macrophages in atherosclerosis development: the role of HSP and LPS

Infected monocytes are responsible for the dissemination of *C. pneumoniae in vivo* both by hematogenous and lymphatic routes (Moazed et al., 1998, Yang et al., 1995). Infection with *C. pneumoniae,* but not with *C. trachomatis* increases adhesion of infected MdMs to non-atherosclerotic vessel walls of healthy mice (May et al., 2003, Kalayoglu et al., 2001). *C. pneumoniae* infection increases trans-endothelial migration of infected MdMs across the blood-brain barrier and they might contribute to the development of sporadic Alzheimer's disease (MacIntyre et al., 2003). In addition, infected monocytes increase the susceptibility of endothelial cells to *C. pneumoniae* infection, but not to infection by *C. trachomatis* or *C. psittaci* (Lin et al., 2000). This could be the mechanism for establishment of vascular wall infection of endothelial and smooth muscle cells, and might subsequently lead to the development of atherosclerosis.

The increased adhesion of infected macrophages to endothelial cells might be due to chlamydial LPS (Kalayoglu et al., 2001). Indeed, chlamydial LPS induces macrophage

foam cell formation, a hallmark in early atheroma development (Kalayoglu and Byrne, 1998). High levels of lipids in blood, low-density-lipoprotein (LDL) in particular, contribute to development of plaques. Chlamydial infection of macrophages activates native LDL to become atherogenic by cellular oxidation and a process that is mediated by chlamydial heat shock protein 60 (cHSP60) (Kalayoglu et al., 1999). cHSP60 is produced in large amounts during persistent infections and co-localises with human HSP60 within macrophages in atherosclerotic lesions. It regulates expression of TNF-α and matrix metalloproteinase (MMP) expression by the macrophages (Kol et al., 1998). Both human and chlamydial heat shock proteins can activate vascular endothelial cells, smooth muscle cells and macrophages through the Toll-like receptor 4 (TLR4) pathways (Kol et al., 2000, Bulut et al., 2002, Sasu et al., 2001). Heat shock proteins induce expression of E-selectin, ICAM-1, VCAM-1 on endothelial cells and secretion of IL-6 by all three cell types. The activation of the cells is abolished with heat treatment, which implies that HSP60 rather than LPS is the major chlamydial component that elicits inflammatory responses, since HSPs are heat labile (Kol et al., 1999). However, very recently there have been reports claiming that HSP-induced inflammatory responses are due to the LPS contamination of recombinant HSP preparations, and that on their own HSPs do not induce any inflammation (Gao and Tsan, 2003a, Gao and Tsan, 2003b). This still needs to be fully investigated and remains an area of controversy.

The role of infected macrophages in *C. pneumoniae* infections supports the importance of *C. pneumoniae* as a cofactor in the development of atherosclerosis.

1.5.5 Dendritic cells

Dendritic cells (DC) are key antigen processing cells and play a central role in linking innate immunity (involving TLRs) and antigen-specific, cytokine stimulated cell-mediated immune responses. *C. pneumoniae* are taken up by dendritic cells but do not multiply within them to produce inclusions. Nevertheless dendritic cells are potently stimulated by *C. pneumoniae* uptake as shown by NFκB activation. There is secretion of IL-12p40 and TNF-α, the expression of MHC class II, CD40, CD80, and

CD86 is up-regulated. The activation of dendritic cells by *C. pneumoniae* is dependent on the presence of TLR2 and independent of TLR4 with the exception of IL-12p40 secretion (Prebeck et al., 2001, Costa et al., 2002). Similar responses are seen with *C. trachomatis*, where infected dendritic cells are activated and produce IL-12 and TNF-α but not IL-10. Following invasion, *C. trachomatis* does not develop into characteristic inclusion bodies. Despite no obvious co-localization between *C. trachomatis* vacuoles and MHC loading compartments, infected DC efficiently present antigens to CD4+T cells and also expand antigen-specific CD8+T cells (Ojcius et al., 1998, Matyszak et al., 2002).

1.5.5.1 Toll-like receptors

Members of the TLR family recognize conserved microbial structures, such as bacterial LPS and viral double-stranded RNA, and activate signaling pathways that result in immune responses against microbial infections. All TLRs activate MyD88-dependent pathways to induce a core set of stereotyped responses, such as inflammation. However, individual TLRs can also induce immune responses that are tailored to a given microbial infection. Thus, these receptors are involved in both innate and adaptive immune responses. The mechanisms and components of these varied responses are only partly understood. Microbial induction of the TLR pathway in dendritic cells blocks the suppressive effect of CD4+CD25+ T regulatory cells, allowing activation of pathogen-specific adaptive immune responses. This block of suppressor activity is, in part, dependent on IL-6 released by dendritic cells (Pasare and Medzhitov, 2003). TLR4 is the best-characterised member of the TLR family and is recognised as the key receptor for the LPS of Gram-negative bacteria. Recently however, there have been reports that different LPS molecules signal through different TLRs. *E. coli* LPS signals through TLR4, but *Porphyromonas gingivalis* LPS signals through TLR2 (Pulendran et al., 2001, Netea et al., 2002). This seems to be a consequence of different conformations of the Lipid A moiety of the LPS.

The activation of immune responses in chlamydial infections seems to depend largely on TLR2; however TLR4 does have a limited role. TLR2 seems to play a role in both

early production of inflammatory cytokines and development of chronic inflammatory pathology in *C. trachomatis* genital infection (Darville et al., 2003). Both *C. pneumoniae* and *C. trachomatis* activate dendritic cells through TLR 2 (Prebeck et al., 2001, Prebeck et al., 2003). On the other hand, purified chlamydial LPS and cHSP60 require TLR4 to activate a variety of cells (Prebeck et al., 2003, Costa et al., 2002, Bulut et al., 2002, Sasu et al., 2001). It is likely that other members of the Toll-like receptor family contribute to the activation of immune responses to chlamydial infections, but this still needs to be elucidated.

1.5.6 T cells

Protective immunity against intracellular bacteria is mostly dependent on the activation of T-cell-mediated immune responses. A number of mouse models were developed to investigate the role of different cells in chlamydial immunity. T cells play a crucial role in all chlamydial infections through Th1-type responses and the production of IFN-γ. In BALB/c mice there is large infiltration of mononuclear cells into the lung during the primary infection, and ~70% of these cells are T cells. During reinfection this is further enhanced and there is a strong Th1-type response with IFN-γ production (Penttila et al., 1998). Similar results were found by Rottenberg et ai (1999). By using a range of mouse knockouts they showed that CD4+T cells initially facilitated the bacterial growth in the absence of CD8+T cells. CD8+T cells hindered the early detrimental effect of CD4+T cells and in later stages of infection both types of cells worked against the infection. The early detrimental effect of CD4+T ceils seemed to be through a Th2 cytokine pattern with high IL-10 and IL-4 mRNA levels and the delayed accumulation of IFN-γ. CD8+T cell-mediated protection was perforin independent and associated with the release of IFN-γ that pushes the CD4+T cell-mediated response to Th1 (Rottenberg et al., 1999, Svanholm et al., 2000). Anti-chlamydial CD8+T cell responses are antigen specific (Wizel et al., 2002). During reinfection the bacterial load is lower, the clearance of the infection is quicker and protection is CD8+T cell-dependent. This was shown by depletion of CD4+ or CD8+T cells. Whereas CD4+T cell depletion did not have an effect on infection kinetics,

depletion of CD8+T cells abolished the protection, reverting the kinetics and bacterial load to the same levels found during primary infection (Penttila et al., 1999).

1.5.7 B cells

Recently, the concept that cell-mediated immune responses alone impart the immunity to intracellular pathogens has been challenged. A number of studies have demonstrated a protective role of specific antibody/B cell responses (Teitelbaum et al., 1998, Edelson et al., 1999, Mittrucker et al., 2000, Elkins et al., 1999, Winslow et al., 2000). This was also demonstrated in *C. trachomatis* mouse pneumonitis lung infections where B-cell-deficient mice showed a higher mortality rate and also a disruption in T cell responses (Yang and Brunham, 1998). B cells also contribute to the outcome of genital *C. trachomatis* infections. CD4+T cells and B cells work in synergy and provide protection from reinfection. B cells also seem to have a crucial role in secondary infections as shown by depletion of both CD4+T and CD8+T cells (Morrison et al., 2000, Morrison and Morrison, 2001). A more recent report implied that B cells participate in anti-chlamydial immunity in genital infections, via FcR-mediated effector functions of antibodies (Moore et al., 2002). The role of B cells has also been studied in *C. abortus* and the results proved similar (Buendia et al., 2002). In the case of *C. pneumoniae* there are very few reports. There is infiltration of B cells into the lung during both primary and secondary infection. On the other hand resistance to *C. pneumoniae* infection does not seem to be associated with increased titres of specific antibodies, since higher levels of anti-OMP2 IgG can be found in IFNγR knockout mice with high infection susceptibility compared to the controls (Rottenberg et al., 1999). However, taking into account all the results available for other chlamydial species there is a high probability that B cells significantly contribute to immunity against *C. pneumoniae*.

1.5.8 Cytokine production and immunity to chlamydial infections

Cytokines play a key role at all levels of chlamydial infections. Chlamydiae are potent inducers of both pro-inflammatory and anti-inflammatory cytokine expression in a number of different cell types. IFN-γ is the key cytokine for control of chlamydial

growth. It has a role both in the early innate responses and in the formation of protective immunity. The initial IFN-γ burst produced by innate cells controls the bacterial load and is necessary for the accumulation of nitric oxide, superoxide production and catalysis of tryptophan, probably related to bacterial stasis/killing (Rottenberg et al., 2000). The protective immunity is mediated by CD8+T cells and is IFN-γ dependent (Penttila et al., 1998, Penttila et al., 1999, Vuola et al., 2000, Wizel et al., 2002).

Production of IFN-γ by bone-marrow derived macrophages seems to be IFN-α/β dependent and IL-12 independent; however, other studies show that lack of early IL-12 production leads to diminished levels of IFN-γ. In turn, IFN-γ is necessary for IL-12p40 accumulation in the lung, suggesting a positive feedback regulation between these cytokines (Rothfuchs et al., 2001, Geng et al., 2000a). In order for IFN-γ to control chlamydial growth *in vitro,* cells need to be pre-treated with the cytokine at inhibitory concentrations (Summersgill et al., 1995). When used in sub-inhibitory doses *in vitro* IFN-γ can contribute to the establishment of persistence (Pantoja et al., 2001). By inducing a persistent state it also changes the gene expression pattern, and there is a marked increase in the expression of MOMP and cHSP60, which can in turn cause a stronger inflammatory response, thus causing immunopathology (Byrne et al., 2001, Molestina et al., 2002). Other cytokines contribute to inhibition of chlamydial growth. TNF-α can synergise with IFN-γ; however, it cannot control the growth independently of IFN-γ in epithelial cells *in vitro* (Summersgill et al., 1995). Other reports claim that TNF-α controls chlamydial growth in monocytes *in vitro* in an IFN-γ independent manner. TNF-β also inhibits chlamydial growth in epithelial cells, but it does so by inducing nitric oxide synthase expression and production of nitric oxide (NO), a powerful bacteriostatic. Another interesting finding is that TNF-β can be added to cell cultures at the time of the infection or even up to 16 hours post-infection, and it still inhibits the growth (Matsushima et al., 1999).

Other cytokines contribute to anti-chlamydial immunity by indirect activation of specific cell-mediated responses. IL-6 is released by infected epithelial cells and

macrophages. Early IL-6 production may contribute to Th1-type immune responses by inhibiting CD4+CD25+ regulatory T cell responses (Pasare and Medzhitov, 2003). On the other hand, IL-10, a cytokine that down-regulates inflammatory responses, is released by infected MdMs. It down-regulates MHC class I expression, and renders the infected cells resistant to apoptosis, and both of these events can modify immune responses and favour the establishment of persistence (Caspar-Bauguil et al., 2000, Geng et al., 2000a). IL-10 knockout mice experience a shorter duration of infection, less microbial burden and limited ascending infection compared to wild-type mice in *C. trachomatis* genital infections (Igietseme et al., 2000). Another regulatory mediator produced in responses to *C. pneumoniae* infection is PGE_2. PGE_2 can inhibit the production of IL-12, IL-6 and TNF-α from macrophages activated with LPS, paired with an increased IL-10 production (van der Pouw Kraan TC et al., 1995, Strassmann et al., 1994). PGE_2 can also suppress IFN-γ production by inhibiting gene transcription and inducing a Th2-type dominant immune response (Katamura et al., 1998).

In summary, cytokines released in response to chlamydial infections contribute to the control of growth, and also to the immunopathology caused by Chlamydiae thereby determining the outcome of the infection.

1.5.9 Factors contributing to chlamydial persistence

From the research to date it is clear that there is a highly complex relationship between Chlamydiae and their hosts, and it is therefore likely that the establishment of persistence in chlamydial infections is a combination of pathogen driven mechanisms and the host immune response. As discussed previously Chlamydiae actively inhibit "digestion" by the host cell and modify the environment in order to set up a successful infection. Apoptosis is an active process of cellular suicide triggered by a variety of physiological and stress stimuli. It is considered a primary defence against intracellular infections and can limit the spread of intracellular pathogens by provoking inflammatory responses and phagocyte activation. Chlamydia-infected cells are highly resistant to apoptosis induced by a variety of stimuli including TNF-α, Fas antibody, granzyme B/perforin, and staurosporine. These factors interfere with many apoptotic pathways through blockade of mitochondrial cytochrome c release or caspase-3, both

crucial points in apoptosis (Fan et al., 1998, Airenne et al., 2002, Greene et al., 2004, Perfettini et al., 2003, van Zandbergen et al., 2004). Apoptosis resistance seems to correlate with IL-10 production from infected PBMC, while in neutrophils it is IL-8 dependent (Geng et al., 2000b, van Zandbergen et al., 2004). Concentrations of IFN-γ that induce the formation of aberrant, persistent Chlamydiae, inhibit apoptosis following *C. muridarum* infection. These results suggest that IFN-γ may contribute to pathogenesis of persistent *Chlamydia* infections *in vivo* by preventing apoptosis of infected cells (Perfettini et al., 2002).

Another pathogen-driven mechanism of persistence might be inhibition of major histocompatibility complex (MCH) activation. MHC-peptide complex expression is crucial for mounting an antigen-specific immune response. MHC class I and class II are recognised by CD8+ and CD4+ cells respectively, and to avoid recognition many intracellular pathogens have evolved strategies for inhibiting MHC expression on the infected cells (Reiner et al., 1987, Schuller et al., 1998). Chlamydiae inhibit MHC expression through a chlamydial protease- or proteasome-like activity factor (CPAF) (Zhong et al., 2001). This factor mediates the degradation of upstream stimulatory factor (USF-1) specific for MHC class II expression and RFX5 downstream transcription factor specific for the IFN-γ-dependent induction of MHC class I expression (Zhong et al., 1999, Zhong et al., 2000). Down-regulation of MHC class I expression is also associated with IL-10 production by the infected cells (Caspar-Bauguil et al., 2000).

MHC molecules can be expressed by non-professional APCs, such as epithelial cells and levels of MHC expression on epithelial cells can be significantly up-regulated by IFN-γ. IFN-γ dependent induction of MHC expression may greatly enhance the ability of the infected cells to present the pathogen-derived peptide on the cell surface for T cell recognition and activation of Th1-type immunity. It seems that the inhibition of the IFN-γ signalling pathways would be a useful way to evade T cell activation. Suppressors of Cytokine Signalling (SOCS) are a group of negative regulators of cytokine signalling that are up-regulated by certain pathogens, and interfere with cytokines and IFN-γ in particular (Stoiber et al., 2001, Imai et al., 2003). The role of

SOCS in relation to IFN-γ and control of chlamydial growth will be discussed in chapter 6.

1.6 *Anti-chlamydial therapy*

1.6.1 Antibiotic treatment

Given the serious implications of a prolonged chlamydial infection there is a growing interest in finding a suitable therapy and developing an effective vaccine to protect against chlamydial infection and prevent tissue pathology. A large number of antibiotics have been tested *in vitro* and *C. pneumoniae* is susceptible to three families of antibiotics: tetracyclins, macrolides and fluoroquinones (Fenelon et al., 1990, Lipsky et al., 1990, Roblin and Hammerschlag, 1998, Strigl et al., 2000, Roblin and Hammerschlag, 2000, Miyashita et al., 2001, Roblin and Hammerschlag, 2003, Hammerschlag et al., 2003, Miyashita et al., 2003). However, *in vitro* results must be assessed by clinical and pharmacokinetic studies by measuring the outcome of the infection in relation to serum, tissue and intracellular levels of different antibiotics. Azithromycin, levoflaxin and clarithomycin are now commonly used to treat *C. pneumoniae* respiratory infections. All three compounds are associated with lower levels of inflammatory cytokines produced by epithelial cells; however they do not eradicate the infection even after prolonged treatment up to 30 days (Kutlin et al., 1999, Kutlin et al., 2002). Azithromycin therapy is associated with decreased cytokine levels and overall reduction of inflammation but the effects are not long-term (Anderson and Muhlestein, 2000, Muhlestein et al., 2000, Semaan et al., 2000). Also, antibiotic treatment does not affect chlamydial growth in infected monocytes, something that can further aid their role in atherogenesis (Gieffers et al., 2001). Therefore, immediate therapy of acute infection may be necessary to prevent the proatherogenic effects of *C. pneumoniae* infection. In the light of these findings early detection is crucial but it is difficult in major part due to the lack of readily available, standardized diagnostic methods. In addition recent studies of serologic and PCR assays for diagnosis of *C. pneumoniae* infection have suggested that there may be substantial inter-laboratory variation in the performance of these tests (reviewed by

(Boman and Hammerschlag, 2002). Until such time as there is a sensitive and reliable test, effective antibiotic therapies of *C. pneumoniae* infections will remain difficult.

1.6.2 Vaccine development

Understanding chlamydial immunobiology gives us important information for vaccine design. The research to date shows that Th1-type T cell-dependent responses convey protective immunity, whereas Th2-type responses are associated with immunopathological responses connected with the worsening of bacterial load and disease severity in mice (Rottenberg et al., 1999). On the basis of this knowledge there are many ongoing attempts to develop an effective and safe anti-chlamydial vaccine. Currently many of these are targeted toward the development of a DNA vaccine based on a protective chlamydial antigen. DNA vaccination is defined as the use of selected genes from pathogenic microorganisms within a eukaryotic expression plasmid. The plasmids contain methylated CpG motifs, which elicit innate cytokine responses that promote Th1-type immune responses. Intracellular synthesis of the microbial protein, especially within transfected professional antigen-presenting cells, facilitates the presentation of antigen on MHC class I and class II molecules and the induction of cell-mediated immunity.

It is crucial to identify the chlamydial antigens that are suitable for use in vaccination. Sequencing the chlamydial genome was a major step in this process. MOMP is the most promising candidate for a vaccine against *C. trachomatis* genital infections. It induces protective immunity when administered in a variety of delivery vehicles and adjuvants (naked DNA; CpG motifs; Freund's adjuvant; lipophilic immune response-stimulating complexes (ISCOMs); MOMP DNA-transfected *Salmonella typhimurium*; recombinant *Vibrio cholerae* ghosts (rVCG)), and delivery routes (oral; intranasal; intramuscular; subcutaneous) (Knight et al., 1995, Pal et al., 1997, Zhang et al., 1997, Igietseme et al., 1998, Brunham and Zhang, 1999, Igietseme and Murdin, 2000, Pal et al., 2002, Eko et al., 2003). Dendritic cells pulsed with recombinant MOMP *in vitro* secrete IL-12 and stimulate T cell proliferation and IFN-γ production. However, when dendritic cells are pulsed *ex vivo* with MOMP and adoptively

transferred to naïve mice, there is a Th2 type, rather than a Th1 type immune response and the mice are not protected from infection (Shaw et al., 2002).

MOMP-based vaccination has also been shown to have protective properties against *C. pneumoniae* infections, but much weaker than the same vaccine for *C. trachomatis.* This could be for a number of reasons, like reduced expression or lower immunogenicity (Murdin et al., 2000). Chlamydial HSP60 is a major activator of cell mediated immunity and is produced in abundance during persistence. Intranasal immunisation of C57B1/6 mice with an hsp60 DNA vaccine induced protection against *C. pneumoniae* that was correlated with higher IFN-γ levels, was CD4+ and CD8+T cell-dependent, and in which no specific antibodies were raised. Intradermal immunisation in the same study caused a strong specific antibody response, but no protection (Svanholm et al., 2000). A cocktail of plasmids containing MOMP, Omp2 and hsp60 was used for intramuscular immunisation of BALB/c mice. Both MOMP and hsp60 vaccination induced a reduction in bacterial load and a strong cell-mediated immunity, but a weak antibody response. Omp2 caused a strong specific antibody reaction, but failed to protect (Penttila et al., 2000).

In addition to DNA vaccines, efforts are also being made to develop vaccines using whole organisms, such as live attenuated vaccines and vaccines using nonviable chlamydial organisms. The advantage of this approach might be that the immunity is developed against all the potential epitopes and could therefore give better protection. Temperature-sensitive mutants of *C. abortus* have been previously shown to be effective in preventing chlamydial abortion in sheep (Rodolakis and Bernard, 1984); however there are no attenuated strains of *C. trachomatis* or *C. pneumoniae*. Su et al developed a surrogate live attenuated vaccine model for *C trachomatis* genital infections by treating the mice with sub-chlamydiacidal concentrations of an antibiotic following an infection. Antibiotic-treated mice generated levels of *Chlamydia*-specific antibody and cell-mediated immunity equivalent to those of control mice, and they showed the same level of protection of control infected mice (Su et al., 2000). The

same group used dendritic cells (DCs) that were pulsed *ex vivo* with heat killed *C. trachomatis* that were then adoptively transferred into mice. DCs produced IL-12p40 and IL-6, and induced *Chlamydia*-specific CD4+ Th1-type immune responses that elicit levels of protective immunity against chlamydial genital tract challenge equal to that obtained after infection with live chlamydial organisms (Su et al., 1998).

Major efforts have been put into development of a chlamydial vaccine, but none of them have proven ideal. Recent advances in chlamydial genomics and proteomics should enhance the chance of identifying putative vaccine candidates and effective delivery systems.

1.7 ***Summary***

In this chapter the current knowledge about chlamydial diseases is presented. Chlamydial infections and related diseases present a growing problem for national health systems worldwide. With the incidence of serious chronic conditions such as atherosclerosis and COPD projected to rise in the near future, it is vitally important to understand the underlying mechanisms of disease development. *C. pneumoniae* has been implicated as a contributory factor to these diseases as well as being recognised as a major cause of respiratory disease. *C. pneumoniae* induces a Th1-type immune response that may contribute to the immunopathology associated with chlamydial infections. However, the mechanism by which *C. pneumoniae* maintains persistent infections and how it manipulates host cell responses are not fully understood. Further investigation is needed to identify these mechanisms and to better understand host-pathogen interactions in order to design novel therapies, and safe and effective prevention strategies. The experiments presented in this thesis focused on investigating the early immune responses following *C. pneumoniae* infections with a view to better understand how *C. pneumoniae* respiratory infections may contribute to the development of chronic diseases.

1.8 ***Hypothesis and Aim of project***

As discussed above, *C. pneumoniae* research has shown that infection causes inflammatory responses, and that prolonged infections may contribute to the establishment and exacerbation of chronic diseases. Based on the research at the time of starting this project, my hypothesis was that the early immune responses of infected lung cells (epithelial cells and MdMs) are responsible for the outcome of the acute infection and contribute to the establishment of persistence. To address this hypothesis, the following studies were pursued:

- The establishment and validation of an *in vitro* model of *C. pneumoniae* infection using a combination of lung epithelial cell lines and primary blood-derived MdMs (Chapter 3)
- Investigation of the cytokine profile induced by infection in lung epithelial cells and MdMs and how these responses are modulated by the treatment of cells with IL-17 (Chapter 4)
- Description of how *C. pneumoniae* infections change the expression of surface molecules on infected cells, and the implications of these findings for the development of immune responses (Chapter 5)
- Investigation of the control of chlamydial growth by inflammatory cytokines and their potential role in the establishment of persistence by the induction of Suppressors of Cytokine Signalling (SOCS) (Chapter 6)
- Preliminary studies into the role of the natural antimicrobial molecules Elafin and SLPI in the control of chlamydial growth. (Chapter 7)

CHAPTER 2

MATERIALS AND METHODS

2.1 *Cell culture and Chlamydia pneumoniae stocks*

2.1.1 General culture conditions

All aspects of cell culture and the handling of *Chlamydia pneumoniae* were performed under biosafety level two conditions. All cell lines and primary cultures were propagated and maintained in antibiotic-free media (unless stated otherwise) in a humidified 5% CO_2 environment at 35°C (Thermo Life Sciences Ltd, Basingstoke, UK). This temperature was chosen to mimic the lung environment. Cell viability was determined by nigrosine exclusion (BDH Chemicals, Poole, UK).

The following human recombinant cytokines were used: IL-17, IFN-γ (R&D Systems Europe Ltd, Abingdon, UK). After purchase they were diluted in IMDM+5%FCS, aliquoted and stored at -70°C until use.

2.1.2 Cryopreservation

Cells were cryopreserved at a density of 1-4x10^6 viable cells/ml in freezing medium which constituted of: 50% of the appropriate culture medium, 40% heat inactivated foetal calf serum (FCS; PAA Laboratories Ltd, Yeovil, UK) and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich Company Ltd, Poole, UK). Cell suspensions were aliquoted into 1ml Nunc Cryotubes (GIBCO BRL, Paisley, UK), cooled to -70°C at a rate of approximately 1°C/minute, using a Nalgene Cryo freezing vessel containing isopropanol (GIBCO BRL), then transferred to liquid nitrogen for long-term storage.

2.1.3 Cell lines and growth conditions

Cells were maintained and propagated in Iscove's modified Dulbecco's medium (IMDM, GIBCO BRL) supplemented with 5% FCS (IMDM + 5% FCS). This medium was used in all cultures unless otherwise stated. The cultures were routinely maintained in 225 cm^2 vented tissue culture flasks (Corning Costar, High Wycombe, UK). Cells were passaged using trypsin/versene (Appendix 9.1.2). All the cell lines used are listed in Table 2.1.

Table 2.1 Details of cell lines used and passage rates.

CELL LINES	TYPE OF CELL			PASSAGE RATE/ WEEK	
	DERIVED FROM	MORPHOLOGY	GROWTH PROPERTIES		
HEp-2	LARYNX CARCINOMA	EPITHELIAL	ADHERENT	1:5	2x
A549	LUNG CARCINOMA	EPITHELIAL	ADHERENT	1:5	2x
NCI-H441	LUNG PAPILLARY ADENOCARCINOMA	EPTHELIAL	ADHERENT	1:5	1x
THP-1	ACUTE MONOCYTIC LEUKAEMIA; MONOCYTE	MONOCYTE	SUSPENSION	1:10	1x
U937	HISTIOCYTIC LYMPHOMA; MACROPHAGE	MONOCYTE	SUSPENSION	1:10	1x

2.1.4 Primary Blood-Derived Monocytes

2.1.4.1 Isolation of blood-derived monocytes

Primary blood-derived monocytes were isolated from anonymous single donor Buffy coats obtained from the Scottish National Blood Transfusion Service after pathogen screening. The Buffy coats were transferred into a 75 cm^2 vented tissue culture flask and diluted 1 in 2 with Calcium-Magnesium-free (CMF) PBS (Appendix 9.1.1). 15 ml of Histopaque-1077 (Lymphoprep^{TM}; Axis-Shield, Norway) was aliquoted into 50ml centrifuge tubes (Corning Costar) and using a pipette, 25ml of Buffy coat/PBS solution was gently layered onto the histopaque, making sure that a clear interface formed between the layers. This mixture was then centrifuged at 1190 g (CR422; Jouan LTD, Ilkeston, UK) at 20°C for 20 minutes without a brake. After the spin, a creamy layer was visible at the interface between the plasma and histopaque. Using a 1.5 ml pasteur pipette, the top section of creamy layer was gently removed and transferred into a new 50 ml centrifuge tube. At the bottom of the interface is where the dead and dying neutrophils are found. CMF-PBS was added to make up 50 ml and the cells were washed twice by spinning at 290g at 20°C for 10min without a brake. After the second wash the cells were pooled into one tube and topped up with CMF-PBS to 50 ml, and then live cells were counted by nigrosine exclusion (1:20 dilution) in a haemocytometer. For every 10^8 cells, 1ml of PBS and 4ml of Red Cell Lysis Buffer (RCLB; Appendix 9.1.4) was added, and the solution was placed on ice for 10 minutes. CMF-PBS was then added to make up 50 ml and cells were washed as before at 290g at 4°C for 10min. The supernatant was discarded; cells were resuspended in 50 ml of CMF-PBS and passed through a 40μm nylon mesh filter (Falcon; Becton Dickinson,

Oxford, UK), to eliminate any clumps. Following the filtration the cells were recounted as above. Yield were typically 0.5-1x10^9 cells per Buffy coat.

2.1.4.2 Purification of Monocyte-derived Macrophages (MDMs)

After the last count cells were washed and resuspended in serum-free IMDM to allow adherence for 2 hrs at 35°C. Cells were used at approximately 2 x 10^6 cells per 1cm^2 of culture surface. After two hours the cells were washed vigorously with PBS two to three times by pipetting the PBS over the cells. After the last wash fresh culture medium (IMDM+5%FCS) was added. Cells were again washed at 24 and 48 hours. The percentage of MdM at 48 hours varied between samples, but it was approximately 70% of total cells as assessed by elastase and GIEMSA staining. After the last wash cells were used for further experiments.

2.1.5 *Chlamydia pneumoniae* AR39

C. pneumoniae AR39 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Tissue culture-derived stocks were obtained by propagation of *C. pneumoniae* in HEp-2 cells. Sub-confluent Hep-2 cells grown in 225 cm^2 vented tissue culture flasks were washed with 5 ml of Hanks Buffered Saline Solution (HBSS; GIBCO BRL) supplemented with 0.2 mg/ml Na_2CO_3. Following the wash the cells were treated with 30 µg/ml of DEAE dextran/HBSS for 20 minutes at RT. After that cells were incubated with 4 ml of IMDM+ 5% FCS containing *C. pneumoniae* at a multiplicity of infection (MOI) of 0.1, for 4 hours at 35°C. The inoculum was pipetted off after 4 hours and cells were supplemented with fresh IMDM +5% FCS containing 1µg/ml cycloheximide (Sigma-Aldrich Company Ltd) and 25µg/ml gentamycin (Sigma-Aldrich Company Ltd). Chlamydiae were harvested on day 3 by disrupting the HEp-2 cells by shaking the culture flask with 3mm diameter sterile glass beads. The resultant suspension was centrifuged at 290g for 10 minutes to remove debris. Supernatants were collected and centrifuged at maximum speed of 16,000g (Biofuge pico; Heraeus, Austria) at 4°C for 20 minutes to pellet the Chlamydiae, which were then resuspended in Chlamydial Transport Medium (Appendix 9.1.4) and stored at -70°C until further use.

2.2 *Polymerase Chain Reaction (PCR)*

2.2.1 RNA extraction

Total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen Ltd, Surrey, UK) according to the manufacturer's instructions. RNeasy technology combines the selective binding properties of silica-gel-based membrane with the speed of microspin technology. A high-salt buffer system allows up to 100 μg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer (lysis buffer **RLT**), which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away (wash buffers **RW1** and **RPE**). RNA is then eluted in 30 μl of RNase/DNase free water. Average RNA content for HeLa cells is approximately 15 μg per 10^6 cells. RNA was stored at -20°C.

Following extraction the quality of RNA was checked by gel electrophoresis. This was done on 1% agarose gels (Appendix 9.1.5) at 100V for 30-45 minutes. The RNA bands correspond to 28S and 18S RNA and were compared to an RNA marker (Ambion Europe Ltd, Huntingdon, UK).

The concentration and purity of RNA was determined using a spectrophotometer (CECIL 2041; Cecil Instruments Ltd, Cambridge, UK) by measurement of UV absorbance. RNA absorbs UV light at 260nm (A_{260}) and protein absorbs at 280nm (A_{280}). Samples were diluted 1:20 in RNase/DNase-free water prior to measurement. RNA concentration was calculated using the following equation:

μg RNA/ml= A_{260} x 20 (DILUTION FACTOR) **x 40** (CONVERSION FACTOR)

Purity was determined by A_{260}/A_{280} ratio. For pure RNA samples this value is approximately 1.8-2 and the values obtained were within this range.

2.2.2 cDNA synthesis

Complementary DNA (cDNA) was synthesised from total RNA using the TaqMan® Reverse Transcription Reagents (Applied Biosystems, Warrington UK). The kit includes: MultiScribe™ reverse transcriptase, RNase inhibitor, dNTP mixture, oligo $d(T)_{16}$, random hexamers, 10X RT buffer, $MgCl_2$ solution. The mix was prepared in 25 µl as follows: 2 µl 10X RT buffer; 4.4 µl $MgCl_2$ solution; 4 µl dNTP mixture, 1 µl random hexamers; 0.4 µl RNase inhibitor; 0.5 µl MultiScribe™ reverse transcriptase. RNase/DNase-free water and 400 ng of total RNA were added to make up 25 µl. The whole procedure was carried out on ice. Reverse transcription was done in Hybaid PCR Thermal Cycler (MBS; Hybaid Ltd, Ashford, UK) using the following programme: 20 minutes at 25°C; 1hr at 48°C; 5 minutes at 95°C; cool down at 4°C. Newly synthesised cDNA was diluted 1.5x with RNase/DNase-free water to obtain the working solution, and stored at -20°C.

2.2.3 Standard PCR

Polymerase Chain Reaction is a standard technique used for amplification of specific DNA sequences by the thermostable Taq DNA polymerase (Chien et al., 1976). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The PCR reaction mixture contained the following components in order: 2.5 µl 10X reaction buffer; 2.5 µl of each 10x primer (100 µM); 2.5 µl of dNTP mix (2mM of each); 0.2 µl (1 U) of Taq DNA polymerase (Roche Diagnostics Ltd, Lewes, UK); 1.5 µl to 2.5 µl of cDNA and RNase/DNase-free water to give a final reaction volume of 25 µl. PCR conditions and genes analysed are listed in Table 2.2.

Table 2.2 Genes analysed by PCR. Two sets of primers were used for SOCS1 and GADPH and three sets for SOCS3 analysis. SOCS3 was also analysed by nested PCR and the cycles are named cycle 1 and 2.

GENE	PRIMER SEQUENCE	PCR CONDITIONS						SIZE IN BP
IDO	FOR 5′-CCTGACTTATGAGAACATGGCGT-3′ REV 5′-ATACACCAGACCGTCTGATAGCTG-3′	1X	30X			1X		**322**
		95° 5MIN	95° 1MIN	50° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	
SOCS1	FOR 5′-CCTGGACGCCTGCGGATTCTACTGG-3′ REV 5′-TCGCGGAGGGGGTTGAGG-3′	1X	30X			1X		**387**
		95° 5MIN	95° 1MIN	57° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	
SOCS1	FOR 5′-GAGAGCTTCGACTGCCTCTT-3′ REV 5′-AGGTAGGAGGTGCGAGTTCA-3′	1X	40X			1X		**491**
		95° 5MIN	94° 30S	58° 30S	72° 30S	72° 10MIN	4° HOLD	
SOCS3	FOR 5′-CTCAAGACCTTCAGCTCCAA-3′ REV 5′-TTCTCATAGGAGTCCAGGTG-3′	1X	40X			1X		**543**
		95° 5MIN	94° 30S	58° 30S	72° 30S	72° 10MIN	4° HOLD	
SOCS3 CYCLE 1	FOR 5′-TCACCCACAGCAAGTTTCCCG-3′ REV 5′-GTTGACGGTCTTCCGACAGAGATGC-3′	1X	25X			1X		**589**
		95° 5MIN	95° 1MIN	57° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	
SOCS3 CYCLE 2	FOR 5′-GAGCCGCCCCCTGGACAC-3′ REV 5′-AGGGGCCGGCTCAACACC-3′	1X	25X			1X		**510**
		95° 5MIN	95° 1MIN	57° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	
GAPDH CYCLE1	FOR 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ REV 5′-CATGTGGGCCATGAGGTCCACCAC-3′	SAME AS TARGET GENE						**983**
GAPDH CYCLE2	FOR 5′-CCACCCATGGCAAATTCCATGGCA-3′ REV 5′-TCTAGACGGCAGGTCAGGTCCACC-3′							**593**
IL-17R	FOR 5′-CTAAACTGCACGGTCAAGAAT-3′ REV 5′-ATGAACCAGTACACCCAC-3′	1X	30X			1X		**833**
		95° 5MIN	95° 1MIN	50° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	
INOS	FOR 5′-GGCCCCACACCCCACCAGAC-3′ REV 5′-GCCAGGCCCGATGAGGATGC-3′	1X	30X			1X		**570**
		94° 4MIN	94° 1MIN	58° 1MIN	72° 2MIN	72° 8MIN	4° HOLD	

2.2.4 Real-time RT-PCR

Real-time RT-PCR is a powerful technique used for detection and quantification of mRNA. It is the most sensitive method for detection and quantification of gene expression levels, particularly for low abundance mRNA, and to elucidate small changes in mRNA expression levels. The technique combines two features: the 5′-3′ exonuclease activity of Taq polymerase and dual-labelled oligonucleotide probes, which only emit a fluorescent signal upon cleavage of the probe, based on the FRET principle (**F**luorescence **R**esonance **E**nergy **T**ransfer). The target-specific probe is labelled with a fluorescent dye (reporter) on the terminal 5′ base and a quenching dye (quencher) at the terminal 3′ base. During the PCR the probe hybridises to an internal region of a PCR product and the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and fluorescence increases in each cycle, proportional to the rate of probe cleavage. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background. This value is defined as the **CT** –threshold cycle.

Generally two quantification methods can be performed: absolute and relative quantification. In absolute quantification the absolute number of mRNA copies is determined by comparison to an appropriate external calibration curve. The relative method is based on the expression ratio of the target gene to a reference gene. The following equation is most commonly used for calculation of the relative expression:

The amount of **target** (gene of interest), normalized to an **endogenous reference** (18S rRNA, GAPDH) and relative to a **calibrator** (internal control-untreated cells), is given by:

$$2^{-\Delta\Delta CT}$$

$$\Delta CT_{target} = (CT_{target} - CT_{ref})$$

$$-\Delta\Delta CT = \Delta CT_{target} - \Delta CT_{calibrator}$$

This is described in detail in ABI PRISM 7700 Sequence Detection System User Bulletin #2 (2001) (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Real time PCR was performed using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All the reagents and plasticware were purchased from Applied Biosystems: Taqman 2x PCR Mastermix, 96 well PCR plates and 8 caps strip and target specific primers and probes. 18S rRNA was used as the reference gene and was labeled with VIC (Vinyl chloride) as the reporter and the target gene probes were labeled with FAM (6-carboxyfluorescein). TAMRA (Tetramethylrhodamine) was used on both as the quencher. All the samples were run in duplicates including negative controls. The PCR mix was prepared in the following order: 25 µl of Taqman 2x Master mix; 14 µl of primer/probe mix (25µmol); 2.5 µl 18S (25µmol); 5.5 µl of DNAse/RNase free water. 23.5 µl was pipetted into each well and 1.5 µl of cDNA was added to make up a total volume of 25 µl. In negative controls 1.5 µl of water was added instead of cDNA. PCR conditions were: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 2-step PCR consisting of 15 seconds at 95°C, 1 minute at 60°C.

The results were analysed using the relative quantification method described above. Primers and probe were designed by Dr Lynn Forsyth (Centre for Inflammation Research, University of Edinburgh) using Primer Express software (ABI Prism, Perkin-Elmer, Beaconsfield, UK). Primer and probe sequences used for analysis of SOCS3 expression are listed in Table 2.3.

Table 2.3 Primers and probes for genes analysed by Real time RT-PCR.

GENE	PRIMER/PROBE SEQUENCES
SOCS3	FORWARD 5'-AGAAGATCCCCCTGGTGTTGA-3' REVERSE 5'-TTCCGACAGGAGAGATGCTGAAGAGT-3' PROBE 6FAM-5'-CCGGCCCCTCTCCTCCAACG-TAMRA-3'

2.3 *Flow Cytometry*

Flow cytometry is an analytical tool that allows the discrimination of different particles on the basis of size and colour. The profile of surface molecule expression on lung epithelial cells and MDMs was analysed by flow cytometry on BD FACSCalibur™ (BD Biosciences) using the CellQuest software (BD Biosciences). From each sample 10,000 cells were acquired in a viable cell gate based on forward and side scatter. Molecules analysed are listed in Table 2.4. All antibodies were used at 1µg/ml.

Table 2.4 List of antibodies used in flow cytometry of surface molecules.

MOLECULE	ORIGIN	CLONE	CONJUGATE	CELLS ANALYSED	SOURCE
CD14	MOUSE	MCA1568	RPE/FITC	MDM	SEROTEC LTD
CD40	MOUSE	MCA1590	FITC	MDM	SEROTEC LTD
CD45	MOUSE	5BI	FITC	MDM	MILTENYI BIOTEC LTD
CD54	MOUSE	MCA1615	FITC	EPITHELIAL, MDM	SEROTEC LTD
CD91	MOUSE	MCA1965	FITC	MDM	SEROTEC LTD
CD119	MOUSE	MCA1450	NONE	EPITHELIAL	SEROTEC LTD
HLA DR	MOUSE	G46-2.6	FITC	EPITHELIAL, MDM	BD PHARMINGEN
HLA ABC	MOUSE	G46-6	RPE	EPITHELIAL, MDM	BD PHARMINGEN
TLR4	MOUSE	MCA2061	RPE	MDM	SEROTEC LTD
IGG1 ISOTYPE CONTROL	MOUSE	NOT PROVIDED	FITC	EPITHELIAL, MDM	SEROTEC LTD
IGG2A ISOTYPE CONTROL	MOUSE	NOT PROVIDED	RPE	EPITHELIAL, MDM	SEROTEC LTD

2.3.1 Flow cytometry analysis of MDMs

Monocyte-derived Macrophages (MDMs) were isolated and cultured as described in section 2.1.4. After the initial culture in serum-free IMDM, cells were washed and replenished with IMDM+15% FCS. After the last wash at 48hrs, cells were treated and collected at four different time points. Prior to collection, detachment buffer (Appendix 9.1.6) was added to the culture medium at 1ml of buffer per 10cm^2 of culture surface and cells were incubated for 30 minutes at 35°C. The cells were collected with a rubber cell scraper (Corning Costar) into 50ml centrifuge tubes and topped up with PBS. Cells were washed once by centrifugation at 290 g for 5 minutes at 4°C, resuspended in cold FACS wash buffer (PBS/2%FCS/0.2%NaN_3) and aliquoted into 96-well round-bottomed plates (Fisher Scientific UK Ltd; Loughborough, UK) at

approximately $2x10^5$ cells per well and washed again as above. A mixture of mouse IgG1 and IgG2a (1:100 dilution of high density culture supernatant of clones VPM20 and 21 respectively; in-house MRI (Dutia et al., 1990)) was added for 15 minutes at 4°C to block non-specific binding. Test antibodies were then added to the wells for 30 minutes at 4°C. Cells were washed twice and resuspended in cold PBS and were analysed immediately after by flow cytometry.

2.3.1.1 Viability of MDMs

Viability of MDMs was determined using 7AAD nuclear dye (7-Aminoactinomycin D; Molecular Probes Europe BV, Leiden, The Netherlands) by measuring fluorescence on the FL-3 channel. A separate sample was stained with 5µg/ml of 7AAD for 30 minutes to set the channel voltage and compensation. To measure viability of all other samples 5µl of 7AAD was added immediately prior to acquisition.

2.3.2 Flow cytometry analysis of epithelial cells

Cells were treated and collected at 4 time points. They were detached using Acutase (Sigma-Aldrich). Cells were washed twice with PBS, Acutase was added (enough to cover the surface) and left for 5 minutes at RT. Cells were collected, washed twice in PBS by centrifugation and aliquoted as above. Biocking of non-specific binding was done either with mouse IgG1/IgG2a mixture as above or with normal rabbit serum (1:200, MRI) depending on the test antibody. Cells were then stained with a primary antibody for 30 minutes at 4°C. All antibodies except anti-IFN-γR were directly conjugated. IFN-γR was detected using two different protocols: 2-step FITC detection or 3-step biotin/streptavidin-APC detection. After the primary antibody FITC- or biotin-conjugated rabbit anti mouse secondary antibody (DakoCytomation UK) was added, and left for 30 minutes. Following that, streptavidin/APC (allophycocyanin; Molecular Probes Europe BV) was added to the biotinylated sample for another 30 minutes. After the final staining, the cells were washed twice as before, resuspended in 1% paraformaldehyde/PBS solution, and left at 4°C until acquisition. Routine viability checks of epithelial cells treated with Acutase showed 90-100% viability.

2.4 *Cytokine analysis*

2.4.1 ELISA

The presence of IL-6 in cell culture supernatants was analysed by Sandwich ELISA (Flexia, BioSource, Nivelles, Belgium) according to the manufacturer's protocol.

Polystyrene 96 well microplates were coated with 100 µl/well of coating antibody diluted in PBS and left overnight in the fridge. The following day excess antibody was washed off and the plates were blocked with blocking solution (5% BSA/PBS/0.1%Tween20 (Polyoxyethylenesorbiton monolaurate; Sigma-Aldrich Ltd)) for 2 hrs at RT. After 2 hrs, 100 µl/well of standard and samples was added along with 50 µl of horse radish peroxidase (HRP)-labelled detection antibody and left for 2 hours at RT with continual shaking. Plates were washed and 100 µl of chromogen tetramethylbenzidine (chromogen TMB) was added per well and incubated for 30 minutes with continual shaking. The reaction was stopped and optical density was measured at 450 nm (MRX Microplate Reader; Dynex Technologies, Ashford, UK). IL-6 concentration was calculated using Revelation computer software (Dynex Technologies).

2.4.2 Cytokine Bead Array

The presence of IL2p70, IL-1β, IL-8, IL-6, IL10 and TNF-α in cell culture supernatants was analysed by BD™ Cytometric Bead Array (CBA) (BD Biosciences, Oxford, UK) according to manufacturer's protocol. CBA is a particle-based immunoassay combined with sensitivity of amplified fluorescence detection by flow cytometry. Six bead populations with distinct fluorescence intensities were coated with capture antibodies specific for IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70. The six bead populations were mixed together to form the CBA which is resolved in the FL3 channel of a BD FACSCalibur™ flow cytometer. The capture beads, PE-conjugated detection antibodies, and recombinant standards or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD CBA Analysis

Software. Statistical model is described in chapter 4 and an example is shown in Appendix 9.4.1.

2.5 *Western Blotting*

Protein expression of SOCS was studied by Western Blot. Cells were collected with trypsin/versene and then washed with PBS twice to get rid of any FCS, and the pellets were then frozen at -20°C. Whole cell lysates were prepared in the following way. Samples were defrosted; 1ml per 10^7 cells of freshly prepared lysis buffer (Appendix 9.1.7) was added; cells were homogenised by pipetting with a 200 µl tip 50 times and left on ice for 10 minutes. Using dry ice the lysates were passed through several (3-5) cycles of freeze/thawing with 10 minute intervals on ice. After the last cycle they were homogenised again as above. The lysate was then transferred into a 1.5ml eppendorf tube and spun down at 16000g for 3 minutes. The supernatants were aspirated and the concentration of protein was determined using the BCA protein assay (Pierce, Perbio Science UK, Tattenhall, Cheshire, UK) according to manufacturer's protocol. Pellets were kept frozen at -20°C. Proteins were separated by electrophoresis using precast 10% Bis-Tris gels in MOPS buffer (Appendix 9.1.8) (NuPAGE, Novex, Invitrogen Ltd., Paisley, UK) at 200V for 90 minutes. Proteins were transferred onto a nitrocellulose membrane (Invitrogen Ltd.) for 3 hours at 70mA per gel using a semidry blot. The membrane was washed 3 times (PBS/0.1% Tween 20) for 10 minutes. It was then blocked for 30 minutes in 5% milk/PBS/0.1%Tween 20 (dried milk powder, Marvel, UK). SOCS1 or SOCS3 antibody (sc-9021 (SOCS1) sc-9023 (SOCS3); Santa Cruz Biotechnology; Insight Biotechnology Ltd., Wembley, UK; (SOCS3) Fusion Antibodies, Belfast, UK) was then added at 0.5 µg/ml and incubated for 1 hour at room temperature followed by 3 washes as above. HRP (horseradish peroxidase) conjugated secondary antibody was added at 0.5 µg/ml was added and incubated for another hour. After the incubation the proteins were detected by ECL (Amersham Biosciences Ltd, Little Chalfont, UK). The membrane was washed, dried and ECL was added for 1 minute. ECL Hyperfilm (Amersham Biosciences Ltd) was exposed to the membrane for different periods of time depending on the intensity of the desired bands. The membranes were stripped of the SOCS antibody to be re-stained for β-

actin using the same protocol as above. Stripping buffer constituents and the protocol for stripping are listed in Appendix 9.1.8.

2.6 *Immunohistochemistry*

For analysis by immunohistochemistry cells were grown on 8-well chamber slides (Labtech, Nalgene Nunc). Cells were rinsed with PBS and then fixed in one of two ways:

(1) With 100% cold acetone for 10 minutes. Slides were then air dried at RT and stored at -20°C until staining. These slides were used to detect chlamydial infection by staining for chlamydial LPS using immunofluorescence.

(2) With 90% acetone/10% methanol for 10 minutes. Slides were air dried at RT and overlaid with 100 µl of 5% polyethylene glycol (PEG)/50% methanol/50% deionised H_2O, to preserve the cellular structure. Slides were left at RT for the PEG to dry out, and were then stored at RT for up to a week. These slides were used for analysis of SOCS expression by DAB detection and co-localisation of chlamydial LPS and SOCS by double immunofluorescence.

2.6.1 DAB Immunohistochemistry

SOCS3 expression was analysed by light microscopy using a DAB colour reagent (Diaminobenzidine; DakoCytomation UK) detection system. Slides overlaid with PEG were first washed in methanol for 5 minutes to dissolve the PEG. This was followed by a peroxidase block with 0.15%H_2O_2/methanol for 10 minutes. Slides were then washed three times with PBS for 5 minutes. After that 2-3 drops per well of avidin block (Vector Laboratories Ltd, Peterborough, UK) were added and left for 10 minutes, followed by three washes as above, and 2-3 drops of biotin block per well added for another 10 minutes. After another round of washing, a final Dako protein block (DakoCytomation) was added for 10 minutes. After that rabbit anti human SOCS3 polyclonal antibody (sc-9023; SantaCruz; Insight Biotechnology Ltd., Wembley, UK) diluted in Dako Diluent (DakoCytomation) to 1:50 to 1:200 (4-1 µg/ml) was added at 100µl per well and left for 30 minutes. Slides were washed five times with PBS, and 100µl per well of secondary HRP conjugated goat anti rabbit antibody at 1 µg/ml

(DakoCytomation) was added and left for another 30 minutes. Slides were washed again as above and 100 µl per well of Avidin/Biotin Complex (Vector ABC RTU; Vector Laboratories Ltd) was added, left for 30 minutes and washed three times. DAB colour reagent (1 drop into 1 ml of buffer; DakoCytomation) was added at 150 µl per well for 5 minutes and washed well another 3-4 times. Slides were transferred into a staining rack. Nuclei were counterstained with haematoxylin for 20 seconds, washed thoroughly under tap water, passed through ethanol of ascending concentration (76%, 95%, 100%) to dehydrate the cells, passed through xylene to clear, and mounted with a diethyl pyrocarbonate (DEPC) permanent mounting medium (Thermo Shandon UK, Runcorn, UK). They were left to dry out overnight and analysed the next day.

2.6.2 Immunofluorescence

2.6.2.1 Single Immunofluorescence

Chlamydial Infection was detected by mouse anti-LPS monoclonal antibody (clone 13/4; in-house (Graham et al., 1995)). Slides were defrosted and washed in PBS for 2-4 minutes at RT prior to staining. Anti-LPS antibody diluted 1:200 was added at 100 µl per well and left for 30 minutes at RT in the dark. Slides were then washed 3 times in PBS for 5 minutes, 10µg/ml of FITC-conjugated rabbit anti-mouse immunoglobulin (DakoCytomation Ltd, Cambridge, UK) secondary antibody was added and left for another 30 minutes at RT. Slides were washed as above and nuclei were counterstained with 2µg/ml of PI (Propidium Iodide, Molecular Probes Europe BV) for 1 minute and washed 3 times. Slides were mounted with 50-100 µl of Mowiol 4.88 (Sigma-Aldrich) mounting medium. They were left in the fridge overnight for the Mowiol to harden and were then analysed on an Olympus BX50 fluorescent microscope using a long pass filter so to detect both PI and FITC at the same time. Photographs were taken on the same microscope using an Olympus PM-20 camera on high sensitivity film (Ektachrome 320T; Kodak).

2.6.2.2 Double Immunofluorescence

For co-localisation of chlamydial LPS and SOCS3, slides were stained by double immunofluorescence and analysed by confocal microscopy (Leica TCSNT confocal

system; Leica Microsystems Heidelberg GmbH, Germany). Slides were immersed in methanol for 5 minutes to remove PEG, washed twice in PBS for 5 minutes and blocked with Dako protein block for 10 minutes. After that 100 µl of mouse anti-LPS (1:200; MRI) was added and left for 30 minutes. Slides were washed 5 times for 3 minutes, and then 1.5 µg/ml of secondary Alexa Fluor® 568 conjugated goat anti-mouse immunoglobulin (red; Molecular Probes Europe BV) was added at 100 µl per well. Slides were washed as above and the same procedure was repeated for SOCS3. Rabbit anti-SOCS3 antibody was added at 1µg/ml, and Alexa Fluor® 488 conjugated goat anti-rabbit immunoglobulin (green; Molecular Probes Europe BV) was added at 1 µg/ml. After the last antibody, slides were washed 5 times as above and nuclei were counterstained with 100 µl of 2 µM To-Pro (blue; To-Pro®3-iodide; Molecular Probes Europe BV) for 5 minutes. Slides were then washed twice with PBS and once with ultra pure water. They were mounted with Mowiol, left in the fridge overnight and anaiysed the next day by confocal microscopy.

CHAPTER 3

IN VITRO MODEL OF *CHLAMYDIA PNEUMONIAE* INFECTION IN THE LUNG

3.1 *Introduction*

The aim of the project was to analyse the earliest immune responses to primary *C. pneumoniae* infection of the lung in humans with a view to understanding how these early responses contribute to long term persistent infections. It would have been desirable to study the host cell-pathogen interactions in primary human lung epithelial cells; however it is becoming increasingly difficult to obtain primary human material for research purposes and gain ethical approval. Taking this into consideration a combination of established human lung epithelial cell lines and primary blood derived monocytes was used to establish a model of early *C. pneumoniae* infection *in vitro*. In this chapter the establishment of the model will be discussed in detail.

3.2 *Experimental Approach*

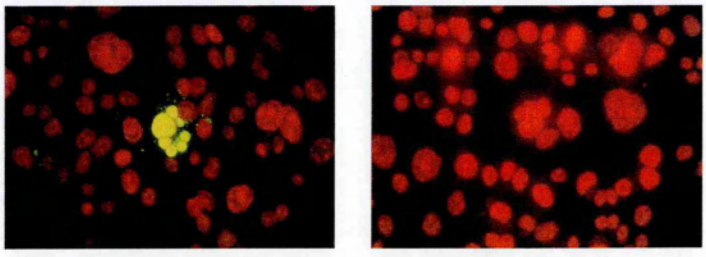
Cell culture conditions, propagation of *C. pneumoniae* and purification of primary blood-derived monocytes are described in detail in section 2.1. Flow cytometry analysis of A549 and HEp-2 cells is described in section 2.3.2 and immunofluorescent staining of infected cells in section 2.6.2.1.

3.3 *Results and Discussion*

3.3.1 Growth of *C. pneumoniae* in transformed lung epithelial cell lines

Host cell phenotype has a bearing on the size and appearance of the inclusions and the rate of *C. pneumoniae* growth. In order to establish a suitable model for the study of early immune responses in lung epithelial cells, three different lung epithelial cell lines were initially considered for this model:HEp-2 cells, derived from a larynx carcinoma, used commonly for *C. pneumoniae* studies; A549 cells derived from a lung carcinoma, a cell line widely used for *in vitro* human lung work, commonly described as a type II pneumocyte (Lieber et al., 1976); and H441 cells, derived from a papillary adenocarcinoma of the lung.

All three cell lines supported *C. pneumoniae* growth; however, H441 cells were later abandoned due to their slow growth (division time around 50 hours) and also a heterogeneous appearance of cells in culture (Figure 3.1). A549 and HEp-2 cells were used in all other experiments and their ability to support *C. pneumoniae* growth is discussed in this section.



infected cells uninfected cells

Figure 3.1 Infection of H441 lung epithelial cells. Cells were seeded in 8 well chamber slides at a density of 10^5 cells/ml and cultured overnight. They were infected with *C. pneumoniae* (MOI=1) and samples were collected 72 hours after infection. Cells were stained for chlamydial LPS (**green**) and nuclei were counter stained with propidium iodide (**red**). Original magnification 200x. Experiment repeated 4x.

HEp-2 cells were used for propagation of *C. pneumoniae* stocks. During propagation of *C. pneumoniae* in HEp-2 cells it was observed that it takes around 72 hours for the inclusions to become visible in culture. Prior to, and after 72 hours (around 96 hours) the inclusions are not visible. At 72 hours there are also some inclusions visible in the

culture medium that were probably released whole from the infected cells. Their number is dependent on the initial multiplicity of infection (MOI) added to the cells. The appearance of inclusions in cell culture is shown in Figure 3.2.

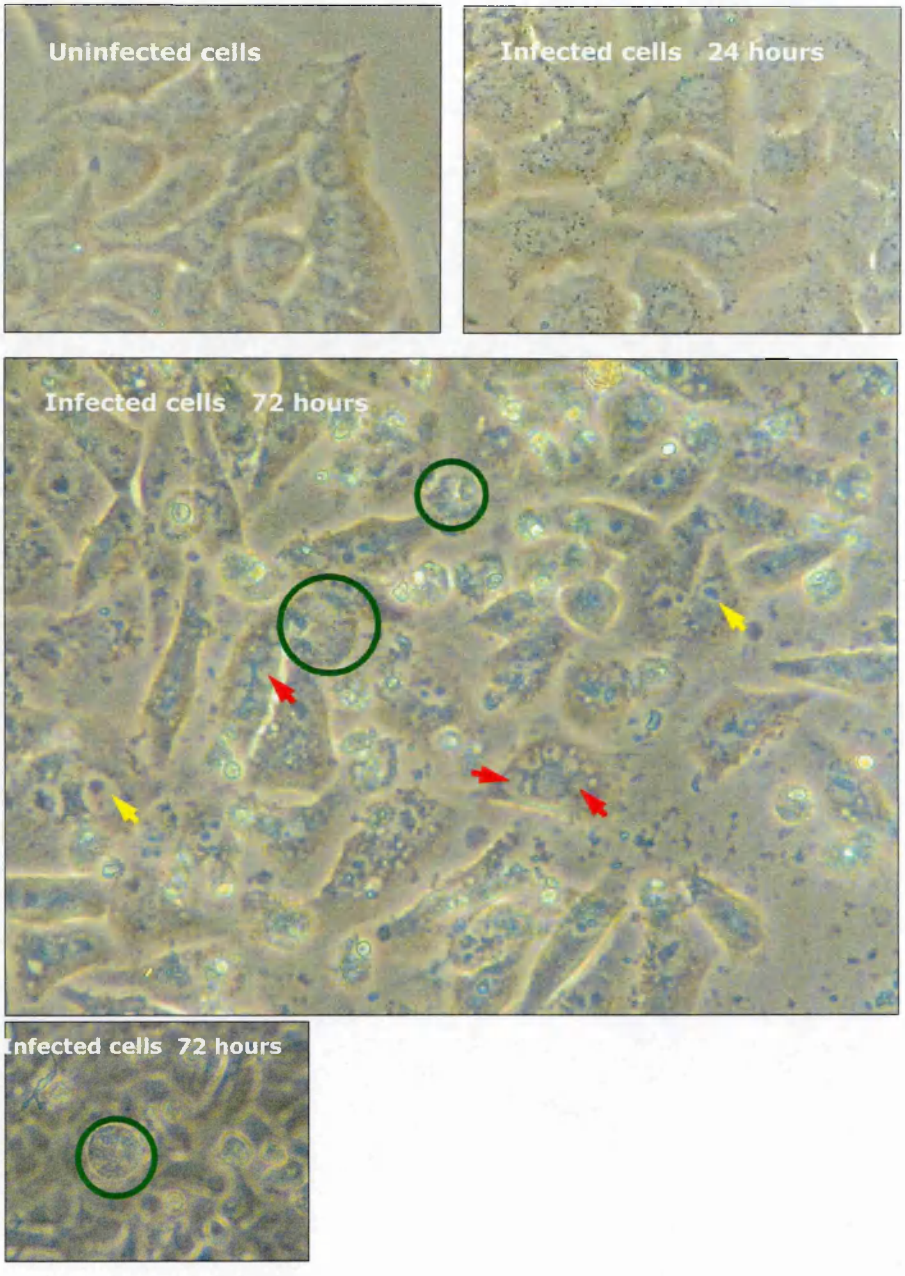


Figure 3.2 Propagation of *C. pneumoniae* in HEp-2 cells over 72 hours. Cells were infected with *C. pneumoniae* (MOI=0.2). Pictures were taken from cells in culture on a phase contrast light microscope. Key: red arrows - inclusions; yellow arrows - nuclei; green circles - floating inclusions. Original magnification 400x. Experiment repeated more than 5x.

During propagation of *C. pneumoniae* stocks HEp-2 cells were maintained in medium containing cycloheximide (1µg/ml) to arrest cell growth and allow more abundant growth of *C. pneumoniae*. Cycloheximide treatment may affect the kinetics of chlamydial growth compared to untreated cells. To ascertain whether there is a

difference, HEp-2 cells were infected for 6 days and kept in cycloheximide-free medium. Samples were collected every 24 hours and stained for chlamydial LPS. The results of this experiment are presented in Figure 3.3. There is no 48 hour sample due to technical error; however the appearance of the cells at that time point was similar to that shown for A549 cells (Figure 3.4 B).

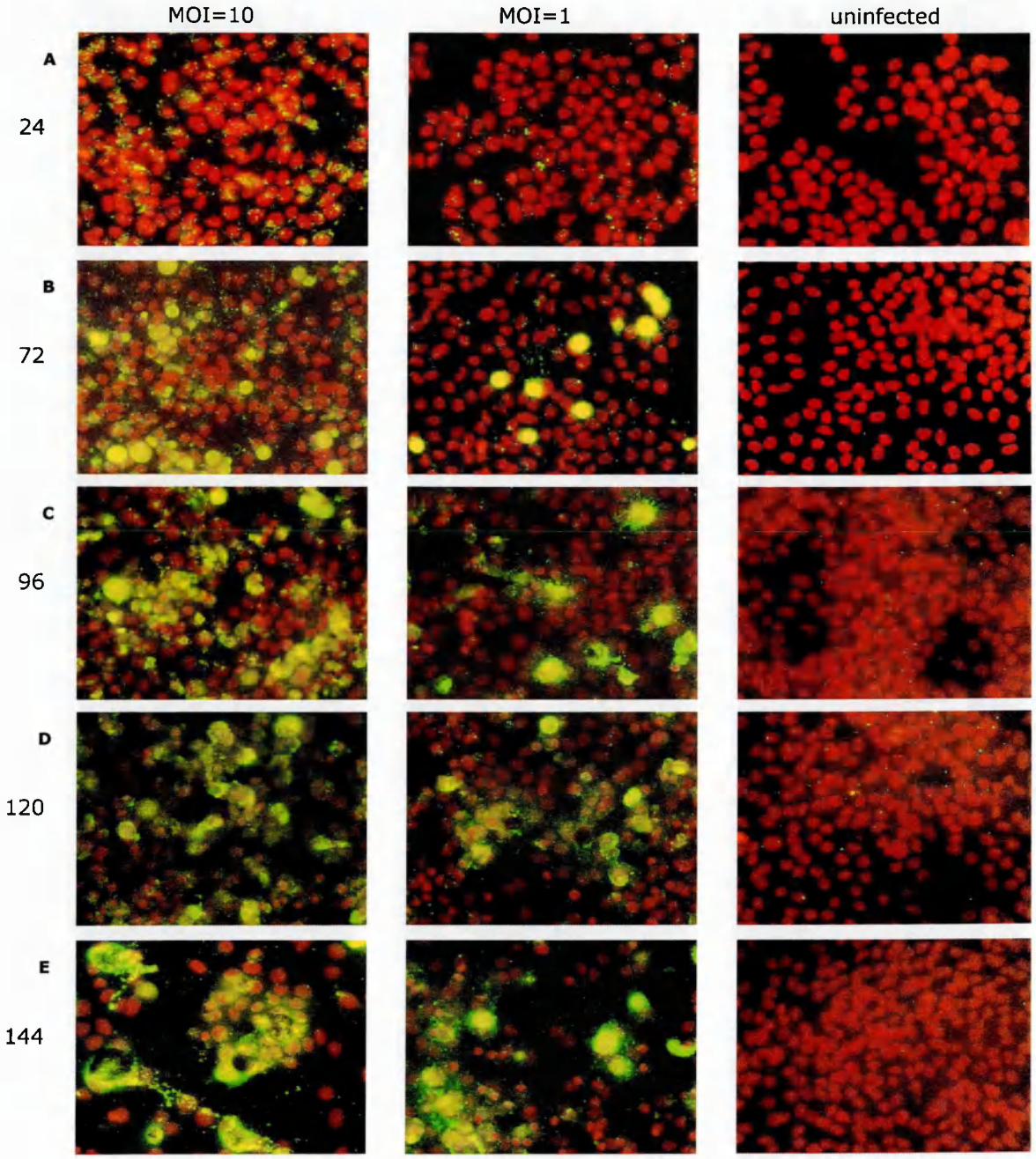


Figure 3.3 Kinetics of infection of HEp-2 cells over 144 hours. HEp-2 cells were seeded in 8 well chamber slides at 10^5 cells/ml and were left overnight. They were infected with *C. pneumoniae* MOI of 1 or 10 and cells were fixed and examined at 24 hour intervals for up to 6 days. Cells were stained for chlamydial LPS (**green**) and nuclei were counter stained with propidium iodide (**red**). Key: **Left column** MOI=10**; Centre column** MOI=1**; Right column** uninfected cells**. (A) to (E)** cells analysed 24 to 144 hours after infection**.** Original magnification 200x. Experiment repeated 3x.

This experiment confirmed that the growth cycle of *C. pneumoniae* is approximately 72 hours in HEp-2 cells. The inclusions develop over 72 hours (Figure 3.3 A and B) such that by 96 hours chlamydial particles are released giving the inclusions a "halo" appearance (Figure 3.3 C). Over the following 48 hours the release of particles continues, and this results in the staining being spread out throughout the HEp-2 monolayer (Figure 3.3 D and E). There is a consistent difference in the number of inclusions and the amount of LPS between the two multiplicities of infection. Also when infected with MOI=1, there are more cells in the monolayer in the later stages of the infection compared to MOI=10, indicating that higher concentrations of *C. pneumoniae* induce more cell death (Figure 3.3 D and E).

The same experiment was repeated with A549 cells and the results are shown in Figure 3.4. A549 cells support chlamydial growth over the entire period of study (144 hours). The appearance of the inclusions and the kinetics of their growth are similar to HEp-2 cells. However there is one obvious difference between the two cell lines and that is the amount of LPS in the later stages of infection. The staining on A549 cells is less "messy" compared to HEp-2 cells throughout the infection. These results show that infected A549 cells release less LPS than infected HEp-2 cells. It has previously been shown that the amount of LPS released by *Chlamydia*-infected cells correlates with the number of inclusions in the cells (Brown et al., 2001). To ascertain whether there is a difference in the amount of LPS released by A549 and HEP-2 cells, the levels of LPS from one experiment (corresponding to the images presented) were analysed by ELISA and those results are shown in Figure 3.5. There was a clear difference in the amount of LPS released from HEp-2 and A549 cells. HEp-2 cells released LPS in the later stages of infection, while A549 cells did not release detectable levels of LPS. The sensitivity range of this ELISA is between 0.78 -100 µg/ml.

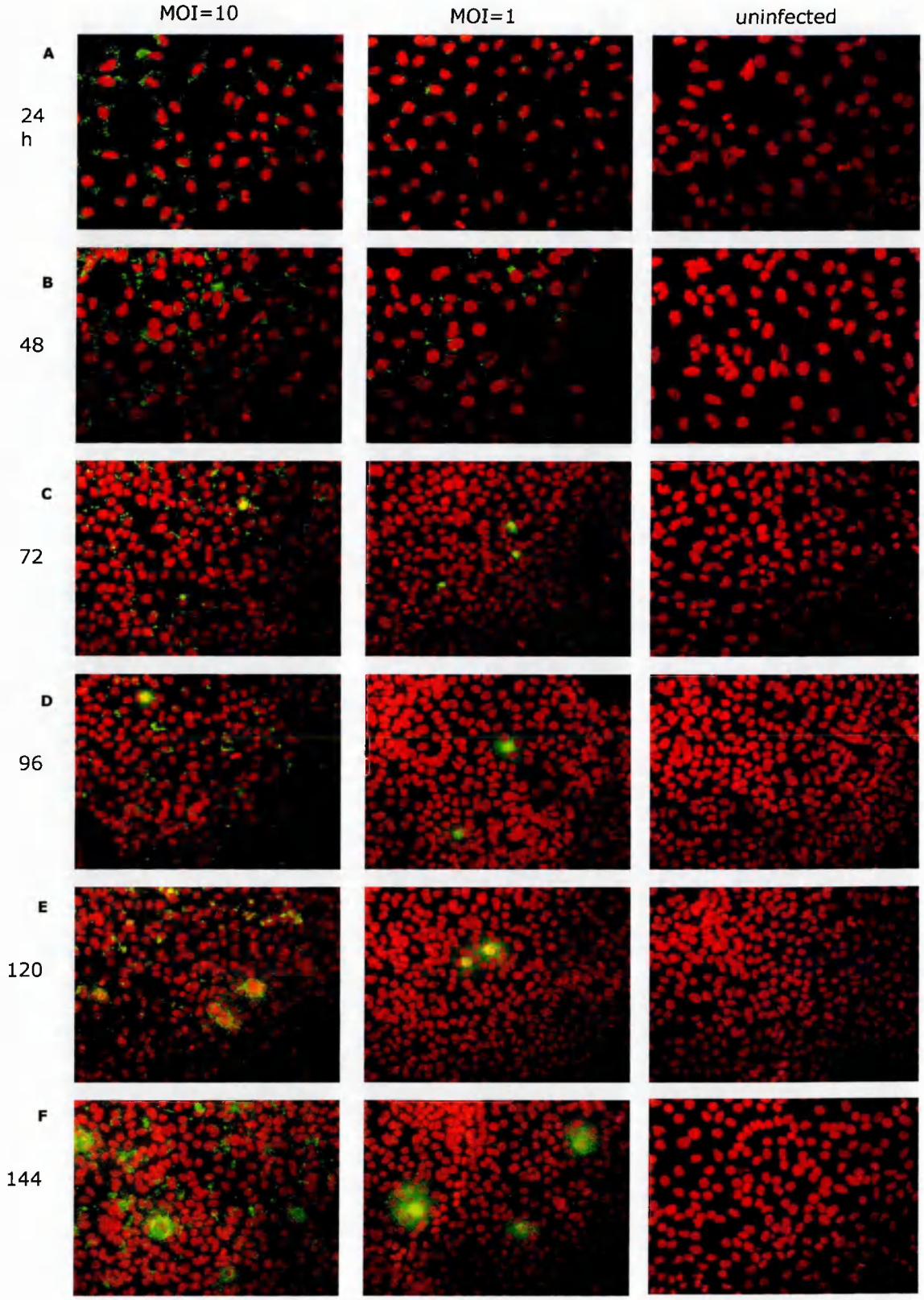


Figure 3.4 Kinetics of infection of A549 cells over 144 hours. A549 cells were seeded in 8 well chamber slides at 10^5 cells/ml and were left overnight. They were infected with *C. pneumoniae* MOI of 1 or 10 and cells were fixed and examined at 24 hour intervals for up to 6 days. Cells were stained for chlamydial LPS (**green**) and nuclei were counter stained with propidium iodide (**red**). Key: **Left column** MOI=10**; Centre column** MOI=1**; Right column** uninfected cells. **(A) to (F)** cells analysed 24 to 144 hours after infection**.** Original magnification 200x. Experiment repeated 3x.

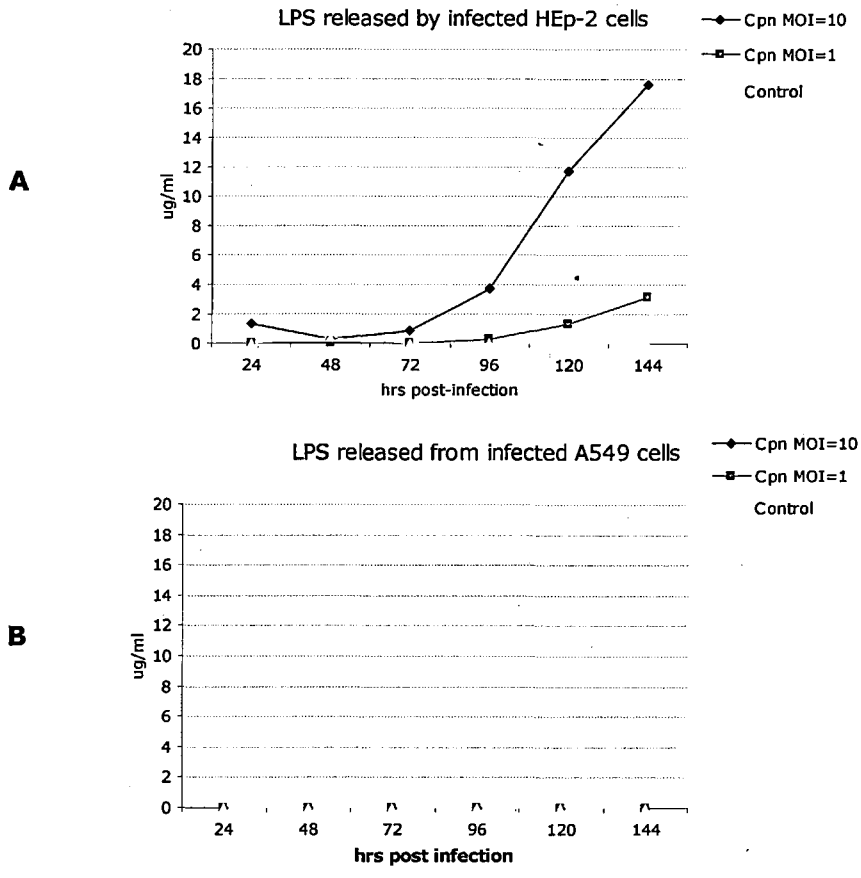


Figure 3.5 Release of LPS by infected lung epithelial cells. **(A)** HEp-2 cells **(B)** A549 cells. Cells were infected with *C. pneumoniae* (MOI=1 or 10) and samples were collected at 24 hour intervals for up to 6 days. This experiment was not repeated; the supernatants analysed correspond to the images shown in images 3.3 and 3.4

Having selected A549 and HEp-2 cells as models of infection, the next step was to perform a similar analysis on monocytes.

3.3.2 Growth of *C. pneumoniae* in monocytes

Initially, two human pro-monocytic cell lines were chosen for analysis of *C. pneumoniae* infections in monocytes. These were THP-1, a commonly used cell line derived from peripheral blood acute monocytic leukaemia, and U937, a pro-monocytic cell line derived from histiocytic lymphoma. Both of these cells grow in suspension and are not adherent. Both cell lines supported *C. pneumoniae* growth as can be seen in Figure 3.6. However these cells were difficult to mature and this presented a technical barrier for the type of experiments I wanted to do with monocytes, so these cells were abandoned in later experiments in favour of primary blood-derived monocytes.

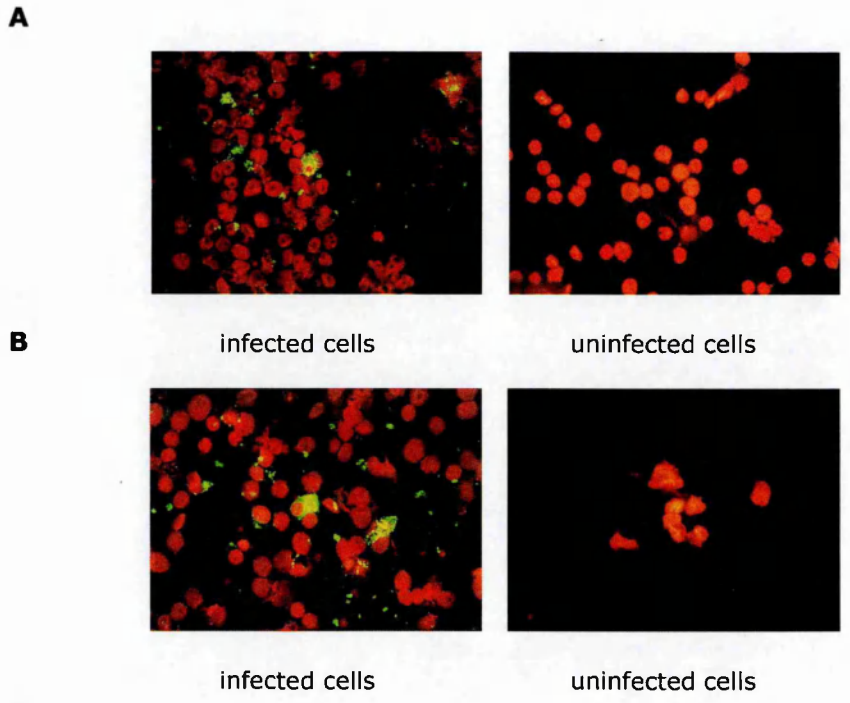


Figure 3.6 Infection of U937 and THP-1 human pro-monocytic cell lines. **(A)** THP-1 cells **(B)** U937 cells. Cells were cultured in suspension at 10^5 cells/ml. They were infected with *C. pneumoniae* (MOI=1) for 72 hours. Cytocentrifuge preparations were stained for chlamydial LPS (**green**) and nuclei were counter stained with propidium iodide (**red**).

As can be seen in Figure 3.7, primary MdMs can support the growth of *C. pneumoniae* for at least 6 days. The fully developed inclusions are seen between 72 and 96 hours (Figures 3.7 C and D) implying a difference in the kinetics of infection compared to lung epithelial cell lines. In later stages, the cells are larger and there is more staining present in the cytoplasm (Figure 3.7. F). However LPS staining in the cytoplasm of infected MdMs is not as marked as in infected HEp-2 cells (Figures 3.3 D and E, and 3.7 F). To confirm this, the amount of LPS released from infected MdMs was measured by LPS ELISA as described above. As can be seen in Figure 3.8, MdMs do not release detectable levels of LPS.

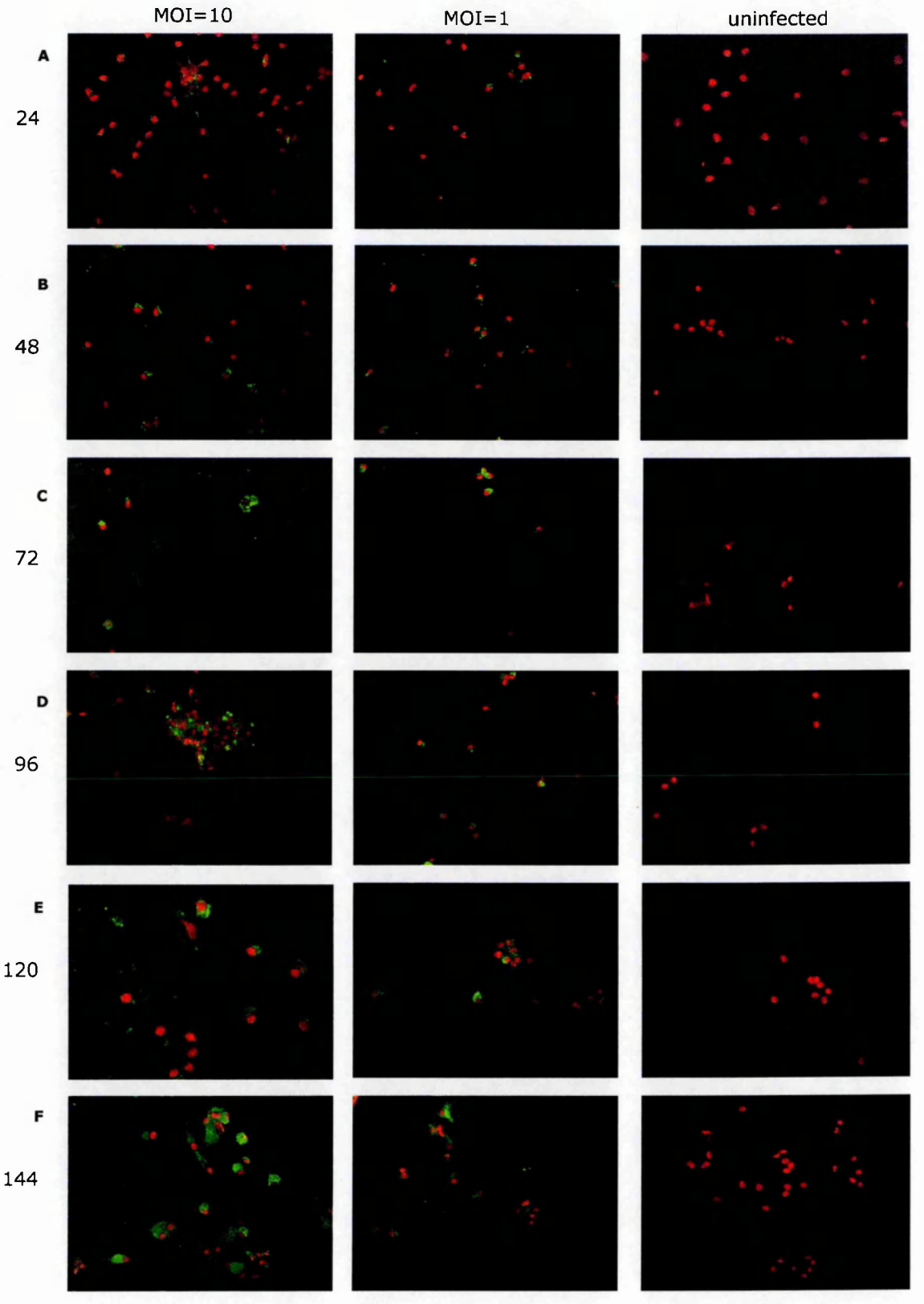


Figure 3.7 Infection of primary blood derived MdMs over 144 hours. Cells were seeded in 8 well chamber slides at 10^6 cells/ml and were cultured for48 hours. After 48 hours cells were infected with *C. pneumoniae* (MOI=1 or 10). Cells were fixed and examined at 24 hour intervals for up to 6 days. Cells were stained for chlamydial LPS (**green**) and nuclei were counter stained with propidium iodide (**red**). Key: **Left column** MOI=10**; Centre column** MOI=1**; Right column** uninfected cells**. (A) to (F)** cells analysed 24 to 144 hours after infection**.** Original magnification 200x. Experiment repeated 3x.

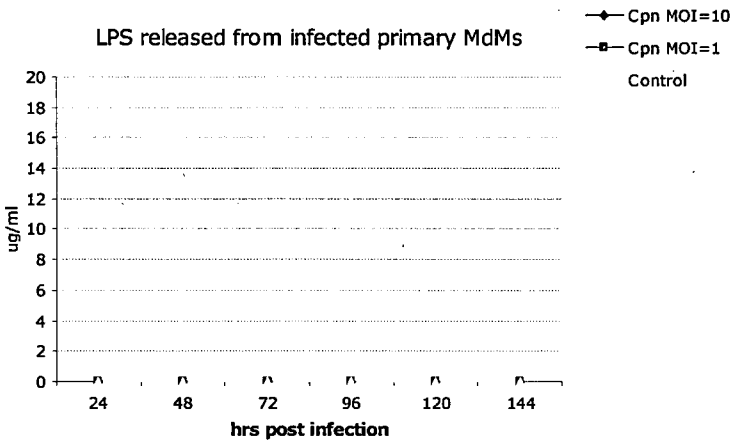


Figure 3.8 Release of LPS by infected primary MdMs over **144 hours.** Cells were seeded in 8 well chamber slides at 10^6 cells/ml and were cultured for 48 hours. After 48 hours cells were infected with *C. pneumoniae* (MOI=1 or 10) and samples were collected at 24 hour intervals for up to 6 days. This experiment was not repeated and the supernatants used correspond to the images shown in the Figure 3.7

In summary, primary MdMs can support the growth of *C. pneumoniae* for at least 6 days; however infected MdMs do not release measurable quantities of LPS. This may also mean that unlike highly susceptible HEP-2 cells, infected MdMs do not release infectious progeny. Other studies have shown that *in vitro* growth of *C. pneumoniae* in monocyte/macrophages is restricted and the amount of infectious progeny released is different. The number of infectious progeny seems to be dependent on both the length of time primary MdMs spend in culture prior to infection, and also on the stage of MdM differentiation. However the number of infectious progeny released by infected MdMs is always lower compared to epithelial cells (Quinn and Gaydos, 1999, Airenne et al., 1999, Kaukoranta-Tolvanen et al., 1996, Kaukoranta-Tolvanen et al., 1996).

3.4 ***Model***

In order to establish a model for this project the following findings were considered:

- All cell types analysed support the growth of *C. pneumoniae*
- *C. pneumoniae* growth cycle in HEp-2 and A549 human lung epithelial cells lasts approximately 72 hours
- *C. pneumoniae* growth cycle in primary MdMs is between 72 and 96 hours long
- There are differences in the amounts of LPS released following infection of different cell types

Taking these facts together the following *in vitro* model was established:

- Human lung epithelial cell lines A549 and HEp-2 and primary blood-derived MdMs
- All responses following infection of human lung epithelial cell lines A549 and HEp-2 were studied within the time of one cycle of *C. pneumoniae* growth, i.e between 72 and 96 hours
- All responses in primary MdMs were studied within one cycle of *C. pneumoniae* growth and this is between 72 and 96 hours

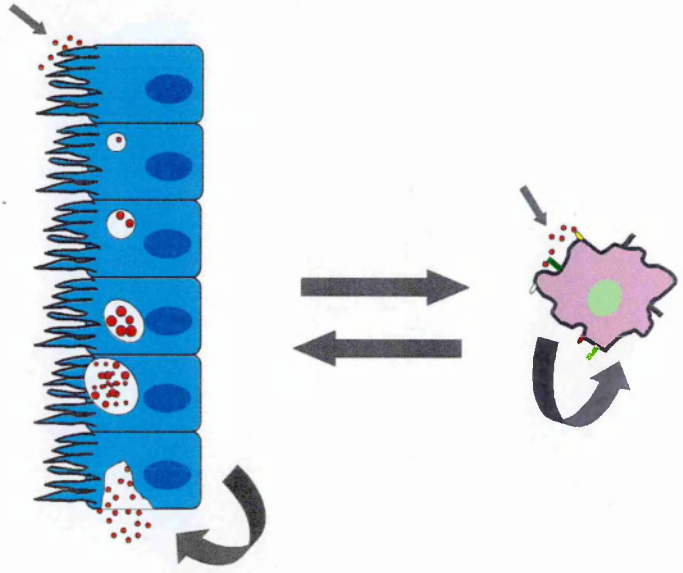


Figure 3.9 *In vitro* model of *C. pneumoniae* infection. This schematic represents possible cell to cell interactions of infected cells. Key: Blue cells – lung epithelial cells; red dots – chlamydial particles; pink cell – MdM; rectangles - cell surface receptors.

The model is presented in Figure 3.9 and it was used throughout the project. A variety of immune responses were analysed and these are presented in the following chapters as outlined in the aims of project section of chapter 1 (page 35).

CHAPTER 4

PROFILE OF CYTOKINES AND CHEMOKINES INDUCED BY *CHLAMYDIA PNEUMONIAE* INFECTION AND MODULATION OF RESPONSES BY IL-17

4.1 *Introduction*

C. pneumoniae infection in the lung induces a pro-inflammatory response that is characterised by the release of pro-inflammatory cytokines and chemokines, and by the infiltration of neutrophils and monocytes soon after infection. The underlying mechanisms of cytokine production, cell recruitment and activation of inflammatory cells are not fully understood. (Molestina et al., 1999)) have shown that infection of endothelial cells with *C. pneumoniae* is followed by IL-8 release and migration of neutrophils into the site of the infection. Lung epithelial cells also produce IL-8 and therefore can contribute to the inflammation. However, it is likely that other inflammatory mediators contribute to the process of the immune response activation. One such mediator with an emerging importance in lung immune responses and lung infections is IL-17.

Recently it has become clear that IL-17 plays a crucial role in lung pathology through its involvement in neutrophil recruitment and activation. Enhanced levels of IL-17 are found in chronic conditions such as asthma and chronic bronchitis, and also in severe acute inflammation (Molet et al., 2001, Barczyk et al., 2003). High concentrations of IL-17 are associated with high neutrophil counts (Laan et al., 2002) and it has been shown that IL-17-mediated neutrophil recruitment and activation is dependent on IL-8 and MIP-2 produced by bronchial epithelial cells and on autocrine production of these chemokines by neutrophils themselves (Laan et al., 1999, Hoshino et al., 2000, Linden et al., 2000, Ferretti et al., 2003). Neutralisation of IL-17 inhibits the neutrophil recruitment associated with LPS challenge (Ferretti et al., 2003, Miyamoto et al., 2003). LPS induces rapid release of IL-17, and in addition IL-17 has been shown to be

crucial for clearance of *Klebsiella pneumoniae* infections in the lung (Ye et al., 2001a). *K. pneumoniae* infection triggers a rapid release of IL-17 as early as 12 hours after infection and the levels are maintained over 48 hours. Mice deficient in IL-17 receptor exhibit higher bacterial load, lower levels of G-CSF and MIP-2 in the lung, and 100% mortality 48 hours after *K. pneumoniae* infection (Ye et al., 2001b). On the other hand, over-expression of IL-17 in the lung by adenoviral transfection is associated with increased neutrophil recruitment and the release of MIP-2, TNF-α, IL-1β and G-CSF as well as enhanced bacterial clearance and survival (Ye et al., 2001a). IL-17 production is also associated with other infections; however its effects have not been extensively studied (Infante-Duarte et al., 2000, Patera et al., 2002).

The principal sources of IL-17 are activated T cells, both CD4+ and CD8+, of both type 1 (IFN-γ) and type 2 (IL-4). However other cells, such as eosinophils, neutrophils and macrophages, can also release IL-17 (Molet et al., 2001, Ferretti et al., 2003, Miyamoto et al., 2003). *K. pneumoniae*-induced release of IL-17 from activated CD4+ and CD8+ T cells has been shown to be TLR-4 dependent and mediated at least in part by IL-23 production from DC (Happel et al., 2003). Whether IL-17 release is TLR-4-dependent in all cells remains to be investigated. IL-17 is a potent inducer of cytokines from different cell types. *In vitro* it can increase the synthesis of IL-11 and IL-6 by bronchial fibroblasts (Molet et al., 2001), the production of IL-6 and IL-8 by bronchial epithelial cells (Kawaguchi et al., 2001) and can activate primary macrophages to release IL-1β, TNF-α, IL-6, IL-10, IL-12 and PGE_2 (Jovanovic et al., 1998).

The role of IL-17 in *C. pneumoniae* infections has not been reported to date; however, it is possible that it plays a crucial role in *Chlamydia*-induced immunopathology. There are a number of parallels to be drawn between IL-17-dependent and Chlamydia-induced immune responses: chlamydial lung infections are associated with early neutrophil recruitment and activation (Yang et al., 1994, van Zandbergen et al., 2004); there is a strong infiltration of T cells (Penttila et al., 1998); and the cytokine

profile induced by *C. pneumoniae* is similar to that of IL-17 treatment.

Taking all of these facts into consideration this study was designed to investigate the following:

- Release of cytokines by infected epithelial cells (A549 and HEp-2) and infected MdMs
- Release of cytokines following IL-17 treatment by epithelial cells and MdMs
- Modulation of immune responses to *C. pneumoniae* infection by IL-17
- Activation of MdMs by infected epithelial cells measured by cytokine production

4.2 *Experimental approach*

Cytokine production by epithelial cells and MdMs was analysed using the Human Inflammation CBA kit (IL-1β, TNF-α, IL-8, IL-6, IL-10 and IL-12; BD Biosciences) The method is described in detail in section 2.4.2 of Chapter 2. A sample standard curve is presented in Appendix B. IL-6 production by MdMs treated with conditioned medium was measured by ELISA as described in section 2.4.1 of Chapter 2. Isolation and culture of MdMs is described in section 2.1.4.

Briefly, A549 and HEp-2 lung epithelial cells were seeded at $2x10^5$ cells/ml per well in 24 well plates in IMDM+5%FCS and left to adhere overnight. MdMs were isolated and seeded at $2x10^6$ cells/ml per well in 24 well plates and left in culture for 48 hours in IMDM+5%FCS. Cells were treated as follows:

- Infected with *C. pneumoniae* at MOI=1 or 10, with or without 50 ng/ml IL-17; samples were collected at different time points up to 96 hours after infection. The experiment was repeated with epithelial cell lines three times, while MdMs isolated from six donors were analysed.
- In a separate experiment the MdMs were treated with 10% conditioned medium (CM) that was prepared from A549 cells infected with *C. pneumoniae* (MOI=1). A549 cells were infected for 24, 48 or 96 hours. The supernatants were collected, filtered through a 0.2μm filter to remove chlamydial particles, and stored at -20°C until use. After thawing, the supernatants were diluted to 10% by adding fresh IMDM+5% FCS. MdMs were treated with CMs harvested at different times and

supernatants were collected 24, 48 and 96 hours after CM-treatment. This was repeated on MdMs isolated from three donors.

Statistical model and analysis

Because of the unbalanced nature of the data the REML directive was used in Genstat 7th edition to fit a model to the data (GenStat Committee (ed.R.W.Payne), 2000). A repeated measures model was used with a uniform correlation structure (subjects were taken as the donor/treatment combinations or experiment/treatment combinations). This is equivalent to fitting a split-plot model with donors designated as blocks, treatments as main plots and time as split plots. The data for each cytokine were positively skewed and so all analyses have been carried out using log transformed data, $\log_{10}(x+1)$.

For the CM experiments, a Student's *t* test was performed and the significance was determined as follows:

P value greater than 0.1	not significant
P value less than 0.05	significant

4.3 *Results*

4.3.1 Cytokine release by lung epithelial cells following *C. pneumoniae* infection and IL-17 treatment

Epithelial cells (A549 and HEp-2) were treated as follows: untreated cells; *C. pneumoniae* MOI=1; *C. pneumoniae* MOI=10; IL-17 50 ng/ml; *C. pneumoniae* MOI=1 + IL-17 50 ng/ml; *C. pneumoniae* MOI=10 + IL-17 50 ng/ml. Production of IL-8, IL-6, IL-10, IL-12p40, IL-1β and TNF-α following the treatments was analysed by CBA. Neither A549 nor HEp-2 cells produced detectable levels of IL-1β, TNF-α, IL-10 or IL-12p40. IL-6 and IL-8 were produced by both cell lines.

The data are presented as both tables and charts of the $\log_{10}$ of the means from three independent experiments, with standard error bars for each data set (Figures 4.1 to 4.4). The raw data are presented in Appendix 3 (Section 9.3 of Chapter 9).

Figure 4.1 IL-6 production (pg/ml) by HEp-2 cells. The values presented are $\log_{10}$ of means from three independent experiments. Error bars in the chart express the SE value. SE=0.05443 Note: values in blue are over the limit of the assay (5000 pg/ml) and were taken as the maximum value. This may bias the data slightly.

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	1.904	2.62	2.027	2.906	2.246	2.835
48 HR	2.013	2.695	2.065	2.878	2.59	3.147
96 HR	2.581	3.144	2.787	3.455	**3.699**	**3.699**

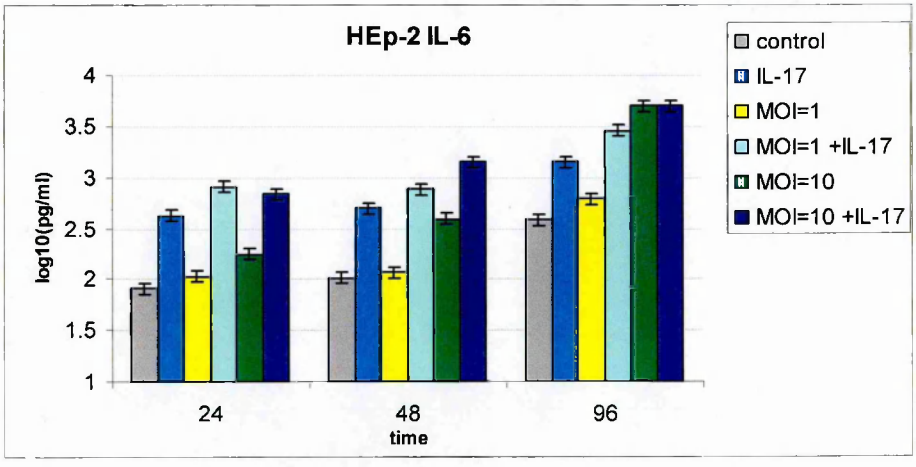


Figure 4.2 IL-8 production (pg/ml) by HEp-2 cells. The values presented are $\log_{10}$ of means from three independent experiments. Error bars in the chart express the SE value. SE=0.09128 **A)** table; **B)** chart. Note: values in blue are over the limit of the assay (5000 pg/ml) and were taken as the maximum value. This may bias the data slightly.

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	0.89	1.185	0.905	1.535	1.179	1.27
48 HR	1.041	1.377	1.035	1.612	1.366	2.025
96 HR	1.332	1.907	1.532	2.375	**3.699**	**3.699**

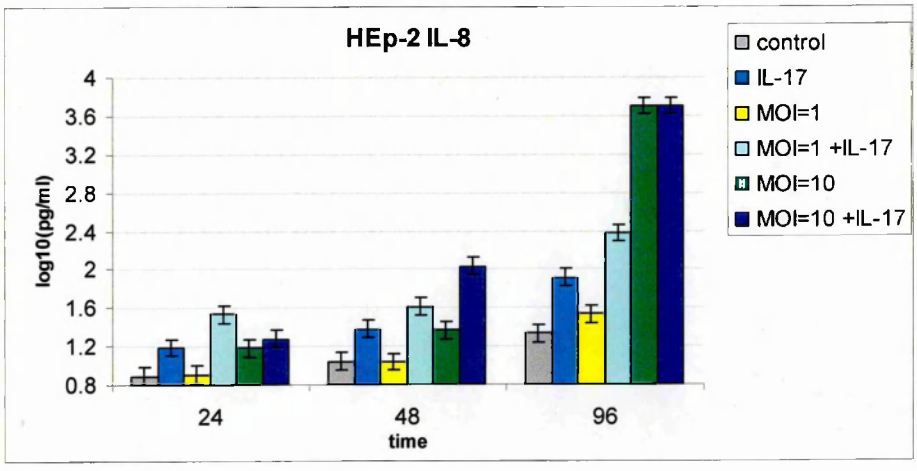


Figure 4.3 IL-6 production (pg/ml) by A549 cells. The values presented are $\log_{10}$ of means from three independent experiments. Error bars in the chart express the SE value. SE=0.1065 **A)** table; **B)** chart

Time	Treatments					
	control	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 hr	0.95	1.08	0.949	1.054	1.595	1.344
48 hr	0.655	0.799	0.896	0.994	1.531	1.538
96 hr	0.828	1.058	1.076	1.054	1.771	1.649

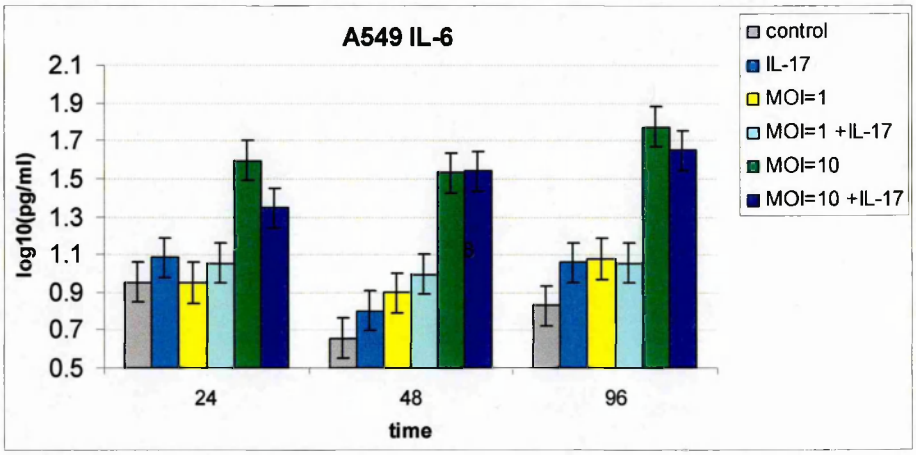
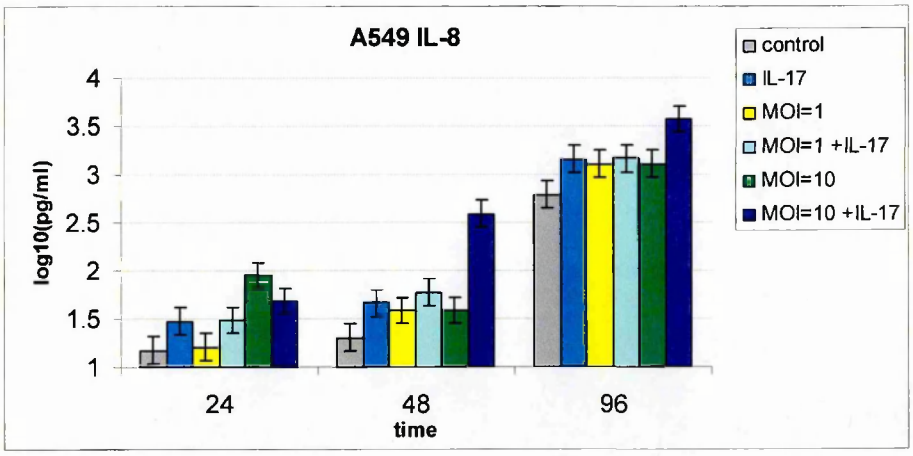


Figure 4.4 IL-8 production (pg/ml) by A549 cells. The values presented are $\log_{10}$ of means from three independent experiments. Error bars in the chart express the SE value. SE=0.1391 **A)** table; **B)** chart

Time	Treatments					
	control	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 hr	1.171	1.472	1.204	1.481	1.952	1.683
48 hr	1.306	1.662	1.581	1.772	1.581	2.589
96 hr	2.787	3.155	3.103	3.162	3.103	3.568



In summary, *C. pneumoniae* induces an inflammatory response in lung epithelial cells by induction of IL-8 and IL-6. Different cells lines preferentially produce different cytokines (HEp-2 produce more IL-6 and A549 produce more IL-8). The production of both cytokines is dose and time dependent in both cell lines. IL-17 treatment of both uninfected and infected cells increases levels of IL-8 both cell lines. IL-6 levels are increased by IL-17 treatment in HEp-2 cells, while in A549 cells there is a downward trend in infected cells.

The same set of experiments was repeated with MdMs and the results are presented in the following section.

4.3.2 Cytokine release by MdMs following *C. pneumoniae* infection and modulation by IL-17 treatment

The production of inflammatory cytokines by MdMs was analysed by CBA. The results are presented as $\log_{10}$ of means from 6 donors, grouped by cytokines and they are presented as both tables and charts (Figures 4.5 to 4.10). A summary of the statistical analysis is presented at the end of this section. The raw data are presented in Appendix 3 (section 9.3 of chapter 9).

Figure 4.5 IL-6 production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.3266 **A)** table; **B)** chart

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	0.716	0.561	3.284	3.189	3.661	3.676
48 HR	0.843	1.343	3.78	3.884	4.438	4.319
96 HR	0.788	0.757	3.848	3.808	4.487	4.501

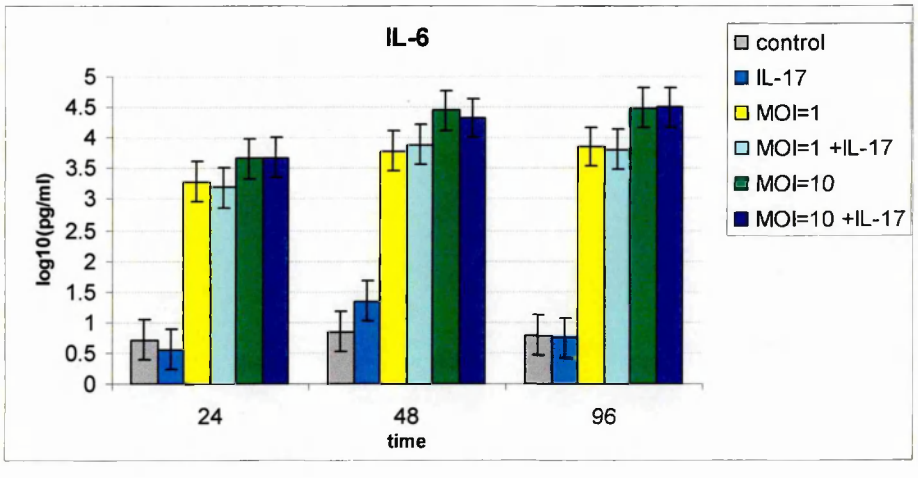


Figure 4.6 IL-8 production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.1286 **A)** table; **B)** chart. Note: values in blue are over the limit of the assay (5000 pg/ml) and were taken as the maximum value. This may bias the data slightly

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10 +IL-17
24 HR	2.845	3.03	4.417	4.458	4.764	4.812
48 HR	2.984	3.546	4.971	4.917	5.422	5.194
96 HR	3.359	3.411	5.054	4.968	5.057	5.046

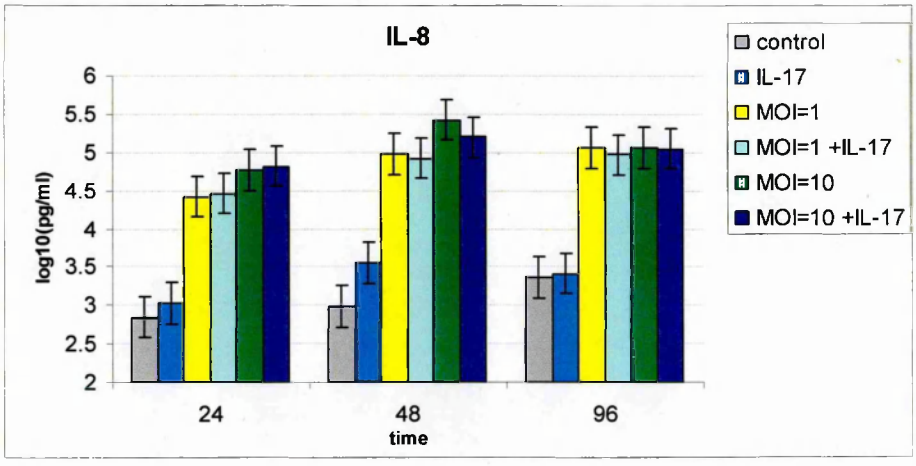


Figure 4.7 IL-10 production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.2010 **A)** table; **B)** chart

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	0.672	0.646	1.581	1.545	2.104	2.137
48 HR	0.678	0.822	1.722	1.619	2.308	2.336
96 HR	0.839	0.631	1.675	1.59	2.214	2.146

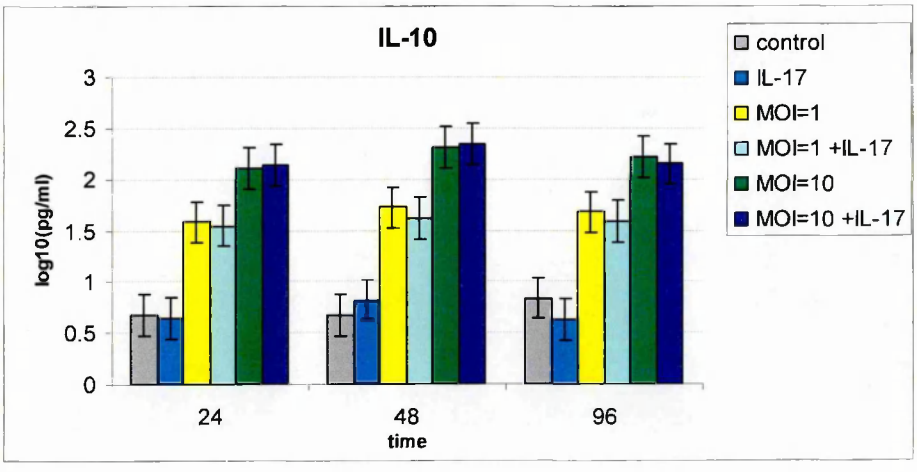


Figure 4.8 IL-1β production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.3669 **A)** table; **B)** chart

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	1.026	0.894	1.411	1.299	2.642	2.522
48 HR	1.243	1.104	1.452	0.939	3.023	2.741
96 HR	0.857	0.916	1.117	0.948	2.891	2.639

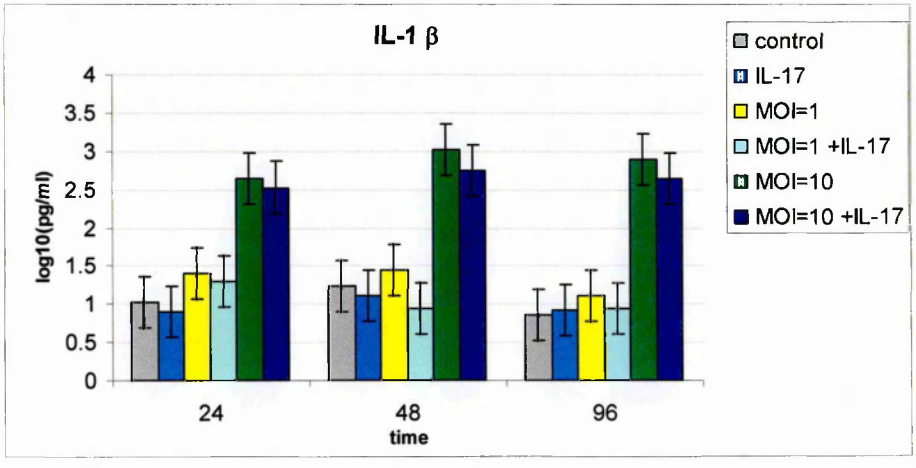


Figure 4.9 TNF-α production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.2353 **A)** table; **B)** chart

TIME	TREATMENTS					
	CONTROL	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 HR	0.503	0.442	2.077	1.99	2.514	2.406
48 HR	0.524	0.545	1.618	1.358	2.556	2.477
96 HR	0.584	0.562	1.097	0.98	2.172	1.98

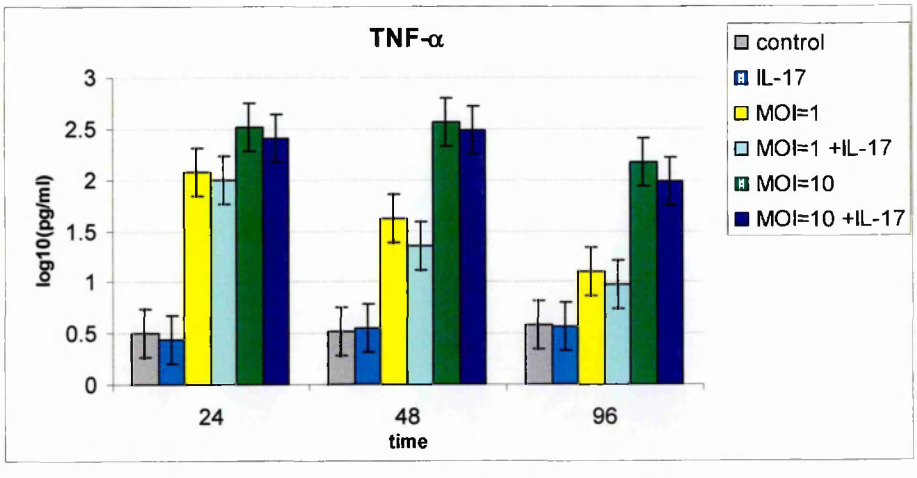
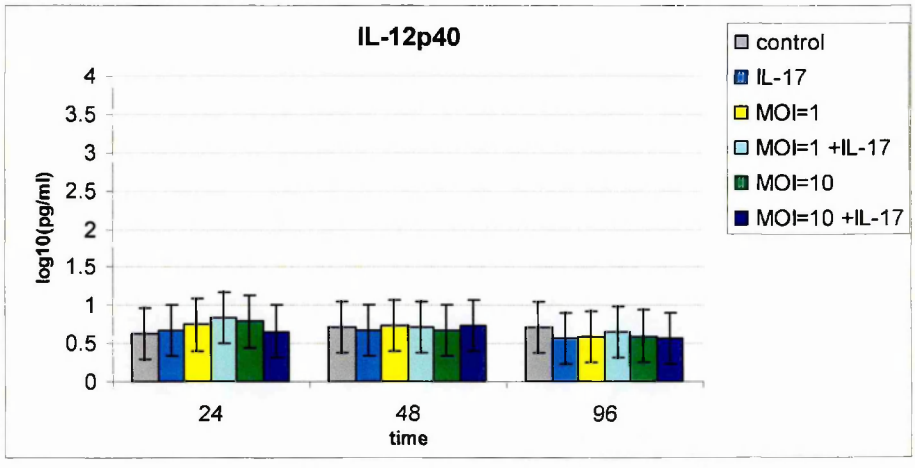


Figure 4.10 IL-12 production (pg/ml) by MdMs. The values presented are $\log_{10}$ of means from six donors. Error bars in the chart express the SE value. SE=0.1203 **A)** table; **B)** chart

Time	Treatments					
	control	IL-17	MOI=1	MOI=1 +IL-17	MOI=10	MOI=10 +IL-17
24 hr	0.6361	0.6627	0.7448	0.8296	0.7865	0.6582
48 hr	0.705	0.6696	0.727	0.7139	0.6639	0.7392
96 hr	0.7109	0.567	0.5906	0.6509	0.5966	0.5719



In conclusion, *C. pneumoniae* infection induces an inflammatory response in the infected MdMs by induction of IL-8, IL-6, IL-1β and TNF-α, but also the regulatory cytokine IL-10. The effect of *C. pneumoniae* is dose dependent for all cytokines except IL-8. Release of IL-8 and IL-6 is increased with time, while TNF-α levels drop after 24 hours. The production of IL-10 and IL-1β is not time dependent. The effect of IL-17 treatment is very limited. In uninfected cells it induces only IL-8 48 hours after infection. Treatment of infected cells shows a down-ward trend in release of IL-8, IL-1β and TNF-α, but this is not significant.

4.3.3 Release of IL-6 by MdMs following treatment with conditioned medium

CM was prepared from A549 cells infected with *C. pneumoniae* (MOI=1) for 24 (CM24), 48 (CM48) and 96 (CM96) hours, and used to activate MdMs in order to investigate how infected epithelial cells may activate alveolar macrophages. IL-6 was chosen as a marker of activation since it is a known product of activated MdMs. The *P* values of the one tailed paired t-test are presented in Table 4.1 and the mean values of IL-6 released by infected A549 cells and CM-treated MdMs are presented in Table

4.2.

Table 4.1 P values of the one tailed paired t test for CM treatment of MdMs. Key: ( ) - not significant; (□) – significant; **bold** – very small values, so probably not meaningful; **CM24** – CM derived from A549 cells infected for 24 hours; **CM48** - CM derived from A549 cells infected for 24 hours; **CM96** - CM derived from A549 cells infected for 96 hours.

TREATMENT	TIME	CM24	CM48	CM96
UNTREATED Vs 10% CM	24	0.2834	0.3575	0.1096
	48	0.1195	0.0724	0.0892
	96	0.2705	0.1269	0.1447

Table 4.2 Means of IL-6 levels in infected A549 cells and CM-treated MdMs. Means for MdMs from three independent experiments; means for A549 from 2 independent experiments. A549 IL-6 levels are from undiluted supernatants. Key: **CM24** – CM derived from A549 cells infected for 24 hours; **CM48** - CM derived from A549 cells infected for 48 hours; **CM96** - CM derived from A549 cells infected for 96 hours.

TIME	A549	CM24		CM48		CM96	
	INFECTED	UNTREATED MDMS	TREATED MDMS	UNTREATED MDMS	TREATED MDMS	UNTREATED MDMS	TREATED MDMS
24	**162.64**	4005.9	4515.9	6406	5756.6	4654.2	5357.9
48	**135.45**	3562	4149.6	3932	4510.8	3761.6	5014
96	**183.11**	3900	4477.1	4137.2	5042.7	3189.2	5814.9

Amounts of IL-6 in A549 cells infected by MOI=1 *C. pneumoniae* measured by ELISA are very similar at all three time points (shown in red). Background levels of IL-6 were high in untreated MdMs. Treatment of MdMs with any of the CMs did not induce a significant increase in IL-6.

4.4 *Discussion*

Production of cytokines following *C. pneumoniae* infection has been shown in a variety of cell types. Epithelial cells, neutrophils, macrophages, DC and T cells all contribute to the cytokine profile and through that to the overall immune response. *C. pneumoniae* induces a largely pro-inflammatory response characterised by induction of mediators such as IL-6, IL-8, TNF-α, IL-1β, IL-12p40 and IFN-γ (Kaukoranta-Tolvanen et al., 1996, Heinemann et al., 1996, Kol et al., 1999, Prebeck et al., 2001, Rothfuchs et al., 2001, Costa et al., 2002, Yamaguchi et al., 2002, Burian et al., 2003, Gencay et al., 2003, Yang et al., 2003, van Zandbergen et al., 2004); however the production of

anti-inflammatory mediators IL-10 and PGE_2 has also been reported (Redecke et al., 1998, Caspar-Bauguil et al., 2000, Jahn et al., 2000). These findings suggest a highly complex network of pro- and anti-inflammatory mediators involved in cross-activation of different cells recruited to the site of infection, and the modulation of their responses. How this network of cytokines contributes to the overall immunity to *C. pneumoniae* infections remains to be clarified.

In the current investigation it was shown that *C. pneumoniae* infection activates lung epithelial cells to release IL-8 and IL-6 but not any of the other cytokines analysed (IL-1β, TNF-α, IL-12p40 or IL-10; Figures 4.1 to 4.4). Primary MdMs are activated to release IL-8, IL-6, IL-1β, TNF-α and IL-10 but not IL-12p40 (Figures 4.5 to 4.10). *C. pneumoniae* was found to exert dose dependent effects. It was also shown that CM derived from infected epithelial cells does not activate MdMs to produce significant amounts of IL-6 (Tables 4.1 and 4.2).

Cytokine production by host cells following infection is one of the first steps in mounting an immune response. However it is difficult to dissect how and in which sequence different host cells activate each other, or how each of the cytokines contributes to the early immune responses. The release of IL-8 may contribute to neutrophil recruitment to the site of the infection and their subsequent activation, while IL-6 may further contribute by activating a Th1 type response by inhibiting CD4+CD25+ regulatory T cells (Pasare and Medzhitov, 2003). TNF-α production by MdMs can contribute to control of chlamydial growth by synergising with IFN-γ as reported by (Summersgill et al., 1995). TNF-α may also contribute to the inflammation along with IL-1β by activation of Th1 type responses.

The most interesting and surprising finding was the release of IL-10 coupled with the lack of IL-12p40 production by infected MdMs. As discussed in Chapter 1, the release of IL-10 by infected cells is associated with down-regulation of MHC class I molecules and an increased resistance to apoptosis by infected cells (Caspar-Bauguil et al.,

2000, Geng et al., 2000). This could contribute to persistent infections. Also, the lack of IL-12p40 production by infected MdMs may decrease IFN-γ production, the key cytokine in the control of chlamydial growth. These findings suggest that *C. pneumoniae* induces such an immune response as to ensure a long-term infection, and this may explain the lack of symptoms seen in chlamydial infections: a balanced cytokine profile and inhibition of controlling mechanisms. Other responses may include modulation of cell-surface molecules and activation of inhibitory mechanisms. These responses were also analysed and are discussed in the following chapters.

To further understand how *C. pneumoniae* activates immune responses, MdMs were treated with CM derived from infected epithelial cells. The initial idea was to use a co-culture system similar to that used in a study on activation of macrophages by epithelial cells infected with *Mycobacterium avium* (Sato et al., 2002). However, I was unable to find a suitable culture system where I could ensure that no chlamydial particles (EBs) could cross the barrier and infect the cells, and this was crucial since the aim was to analyse activation by mediators released from infected cells and not by infection. For that reason, it was decided to use a conditioned medium. Epithelial cells were infected; supernatants were filtered to remove chlamydial particles (EBs) and then used at 10% of original concentration. 10% CM was used for a number of reasons, principally the amount of medium needed in total and the need to have a sufficient amount of fresh medium to maintain the cells. The production of IL-6 was chosen as a marker of activation since it is a known product of activated MdMs. CMs derived from three time points (24, 48 and 96 hours of infection) were used, and none of them induced significant levels of IL-6. However, this finding does not mean that the cells were not activated, and other markers of activation might give a different result. Future studies could include a more comprehensive analysis, including other cytokines such as IL-8, IFN-γ, GM-CSF, IL-1β, TNF-α, IL-10 and possibly others; a higher MOI could be used in the initial infection of epithelial cells; and also different CM concentrations may give a different effect. In addition to measuring IL-6 production following CM-treatment, modulation of cell-surface molecules was also

analysed and this is discussed in the next chapter.

As discussed above IL-17 is a cytokine with a growing importance in lung pathology and lung infections, as shown by Ye *et al* (2001) who demonstrated its importance in the down-regulation of inflammatory responses and clearance of *Klebsiella pneumoniae*. It was also shown that IL-17 is important in neutrophil recruitment following LPS exposure of the lungs (Linden et al., 2000). In the present study it was shown that IL-17 up-regulates the release of IL-8 and IL-6 by *C. pneumoniae*-infected epithelial cells (Figures 4.1 to 4.4), while it causes a down-ward trend in the release of IL-8, IL-1β and TNF-α by infected MdMs, but this is not significant (Figures 4.6, 4.8 and 4.9). IL-17 treatment alone also induced IL-8 and IL-6 production in both epithelial cell lines and IL-8 production in MdMs soon after treatment. These findings suggest that IL-17 present in the lung may play a role in neutrophil recruitment activation of the early responses, but may also prevent excessive inflammation following infection. Future studies could be transferred into a mouse model with over-expression of soluble IL-17 receptor or by introducing a blocking antibody, or the other extreme, over-expression of IL-17. In both cases the study could investigate whether the animals demonstrate any change in duration of infection, bacterial burden or cytokine production. That approach would offer a more complete picture into the role of IL-17 in *C. pneumoniae* infection.

As stated in Chapter 2, the purity of the primary blood derived MdMs, was around 70%. The major contaminant cell population were neutrophils and this is likely to affect the cellular responses including cytokine production. One known product of neutrophils that was analysed in this study is IL-8. How the presence of neutrophils affects the production of cytokines is difficult to dissect; however they may increase the total amount of IL-8 released and affect the production of other cytokines in other ways. Also, given the role of IL-17 in neutrophil-mediated responses in lung pathology, effects of IL-17 may also be skewed by the presence of neutrophils.
However, this does not render the results obtained with primary cells less valuable;

this just needs to be kept in mind when analysing and presenting the results.

In summary, this investigation has shown that *C. pneumoniae* induces a pro-inflammatory response in infected cells characterised by the production of inflammatory cytokines and chemokines. However, this is balanced by the release of IL-10 from infected MdMs. Also, the production of inflammatory cytokines is down-regulated by IL-17 treatment, indicating that this cytokine may play a crucial role in chlamydial immunopathology by dampening the inflammation induced in infected cells.

CHAPTER 5

MODULATION OF SURFACE MARKER EXPRESSION ON LUNG EPITHELIAL CELLS AND MDMS FOLLOWING *C. PNEUMONIAE* INFECTION

5.1 *Introduction*

Cells can communicate by releasing mediators or altering the expression of cell-surface molecules to indicate they are infected and thereby promote an effective host immune response. In the previous chapter, profiles of chemokines and cytokines released by lung epithelial cells and MdMs following *C. pneumoniae* infection were presented. To investigate whether *C. pneumoniae* infection alters the expression of cell-surface molecules, a total of 8 cell-surface markers were chosen for analysis on MdMs and lung epithelial cells. These markers include molecules found on MdM surface (CD45, CD14); receptors involved in pathogen recognition (TLR4, CD14); receptors involved in the activation of adaptive immune responses (MHC class I and II molecules, CD40, CD45, CD91) and an adhesion molecule (CD54/ICAM 1). All may contribute to the immunopathology following *C. pneumoniae* infection and their potential importance is discussed below.

It has been shown that Chlamydiae activate a variety of cell types through the Toll-like receptors, TLR2 and TLR4 in particular (Darville et al., 2003, Prebeck et al., 2001, Bulut et al., 2002, Netea et al., 2002, Prebeck et al., 2003). The activation of TLR4 is mediated through ligation of CD14 on the surface of APCs, and *C. pneumoniae* has been shown to up-regulate CD14 expression on infected MonoMac 6 human macrophage cells (Heinemann et al., 1996). Both chlamydial LPS and cHSP have been shown to activate cells in a CD14/TLR4-dependent manner (Kol et al., 2000, Sasu et al., 2001, Costa et al., 2002, Prebeck et al., 2003). In the last few years cHSP, rather than chlamydial LPS, has been identified as the major chlamydial component responsible for eliciting inflammatory host immune responses (Kol et al., 2000). More

recently new research suggests that this may be a result of LPS contamination of the samples used to activate the cells, and not a true HSP-dependent effect; however this is still a controversial area of research (Gao and Tsan, 2003).

CD91 has recently been recognized as a common HSP receptor on APCs. HSP-chaperoned exogenous antigens are delivered to APC that in turn present these through MHC class I thereby activating adaptive immune responses (Binder et al., 2000, Basu et al., 2001). Additionally, HSPs released from cells as a result of stress or necrotic death activate APCs and, as these are highly conserved from bacteria to mammals, the ability of HSP to activate APC provides a unified mechanism for response to internal and external stimuli (Basu et al., 2000, Li et al., 2002). Considering the importance of cHSPs in the activation of an immune response and the role of CD91 as a common receptor, it is possible that CD91-dependent signals play an important role in the establishment of anti-chlamydial immunity. Another potential role of cHSPs released during infection is the up-regulation of adhesion molecules including E-selectin, VCAM-1 and CD54/ICAM-1 as demonstrated by Kol et al., (1999), a process that may contribute to atheroma development.

Activation of the innate immune system via pathogen-recognition receptors/pathogen-associated molecular patterns (PRR/PAMPs) is one of the first steps in mounting an immune response. This is followed by activation of the acquired immune system for generation of specific immunity and immunological memory. An important component of this is antigen presentation and activation of T cells. The ability of MdMs to activate T cell responses may be affected by chlamydial infection. *C. trachomatis* can down-regulate both MHC class I and II molecule expression in a variety of human cells (Zhong et al., 2000, Zhong et al., 1999). *C. pneumoniae* can also down-regulate MHC class I expression on human monocytes, while it up-regulates the expression of MHC class II, CD40, CD80 and CD86 in infected dendritic cells in mice (Redecke et al., 1998, Caspar-Bauguil et al., 2000, Prebeck et al., 2001).

Collectively these observations suggest that Chlamydiae modulate expression of surface receptors. As discussed in Chapter 4, *C. pneumoniae* infection activates host

cells to release inflammatory mediators. Chlamydia-derived proteins such as cHSP, Inc or LPS could also be released. These mediators might in turn alter the expression of surface molecules on infected cells, thereby influencing cell to cell communication and activating the host immune response. To investigate the effect of *C. pneumoniae* on the expression of cell-surface molecules, the following questions were addressed:

- Does *C. pneumoniae* infection of lung epithelial cells change the expression of CD54, HLA ABC, HLA DR or CD119 (IFN-γ R)?
- Does *C. pneumoniae* infection of MdMs alter the expression of CD14, CD40, CD45, CD54, CD91, HLA ABC, HLA DR or TLR4?
- Does conditioned medium from infected epithelial cells alter the expression of cell-surface molecules on MdMs?

5.2 ***Experimental approach***

The expression of CD14, CD40, CD45, CD54, CD91, HLA ABC, HLA DR and TLR4 was analysed by flow cytometry on primary blood derived monocytes, and the expression of IFN-γR, HLA ABC, HLA DR and CD54 was analysed on human lung epithelial cell lines A549 and HEp-2. Epithelial cell lines were cultured as described in section 2.1. The isolation and culture of MdMs is described in section 2.1.4. Flow cytometry protocols and antibodies used are described in section 2.3.

Epithelial cells were seeded in IMDM+5%FCS in culture flasks and left overnight to adhere. The following day they were infected with *C. pneumoniae* (MOI=1) and cells were collected 6, 24, 48 and 72 hours after infection.

For analysis by flow cytometry MdMs were handled as follows. After 48 hours of culture in IMDM+15% FCS with no other stimuli, the following treatments were added:

- *C. pneumoniae* (MOI=1) for 6, 24, 48 and 72 hours. This was repeated on MdMs derived from 4 donors.
- 10% CM from A549 epithelial cells infected with *C. pneumoniae* (MOI=1) for 24 hours. The supernatant was collected, filtered through a 0.2μm filter to remove infectious EBs, and frozen at -20°C until use. After thawing, the supernatant

was diluted to 10% in fresh IMDM+15% FCS. MdMs were collected 6, 24, 48 and 72 hours after culture in CM. This was repeated on MdMs derived from 3 donors.

All the samples were stained for CD14 and one other surface molecule, together with the viable cell stain 7AAD. The results were analysed by gating the CD14 +ve live cell population to present CD14 positive cells expressing the target molecule.

An example of how CD14 +ve cells were gated for analysis is shown in Figure 5.1. First, dead cells were excluded by 7AAD staining in a 7AAD/FSC plot (R1); gated, viable cells were then presented on a FSC/SSC plot and a new gate (R2) was drawn around the monocyte population. The final gate (R3) was set up on a CD14 histogram to represent CD14 positive cells. Finally, all three gates were combined (R1+R2+R3) to present the target surface molecule in a histogram. Cells from 7 donors were analysed and the results for all individual donors are presented.

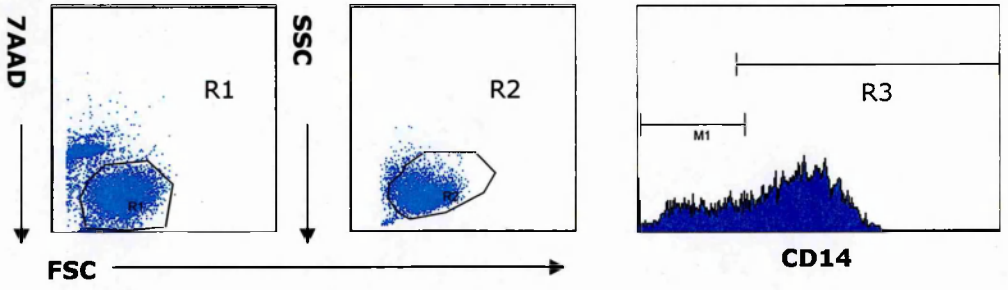


Figure 5.1 An example of gating on CD14 expressing MdMs for analysis of other surface molecules. Key: FSC - forward scatter, SSC – side scatter. R3=R1+R2

5.3 *Results*

5.3.1 *C. pneumoniae* infection of lung epithelial cells does not alter the expression of cell-surface molecules

The expression of HLA ABC, HLA DR, CD54 and IFN-γ receptor α-chain (CD119) on HEp-2 and A549 lung epithelial cell lines was analysed by flow cytometry. The results are presented below in Figures 5.2 to 5.5. Tables with mean fluorescence intensity values (MFI) for HLA ABC and CD54 following infection are presented with the histograms in Figures 5.4 and 5.5.

Both A549 and HEP-2 cells express IFN-γ receptor α-chain (CD119; Figure 5.2) in the resting state. Modulation of IFN-γ receptor expression following *C. pneumoniae* infection is discussed in detail in chapter 6 in the context of chlamydial control.

Neither cells line expresses HLA DR (MHC class II; Figure 5.3) but they both express HLA ABC (MHC class I; Figure 5.4) HLA ABC expression is not affected by chlamydial infection in either A549 nor HEp-2 cells (Figure 5.4), while the levels of CD54 are very low and change is not detectable (Figure 5.5).

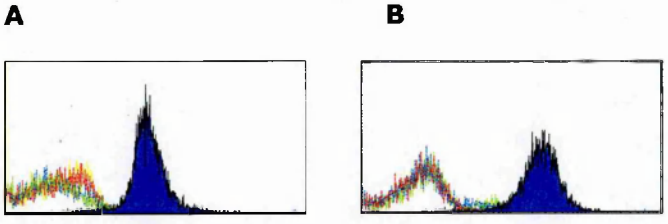


Figure 5.2 The expression of IFN-γR (CD119) on uninfected lung epithelial cells. **(A)** HEp-2 cells **(B)** A549 cells. Key: **purple filed** - IFN-γ R; **blue line** - cells only; **green line** - 1st step control; **red line** - 2nd step control; **yellow line** - 3rd step control.

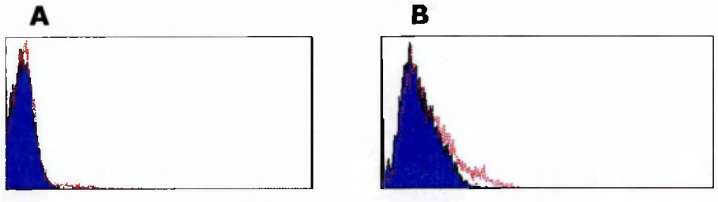
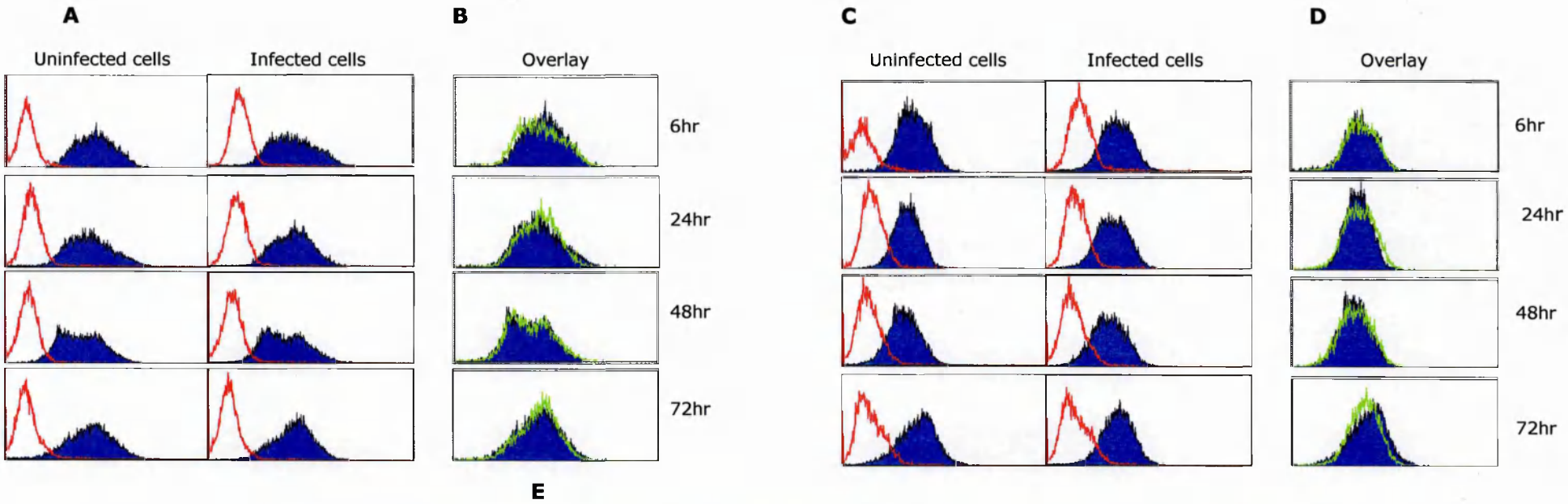


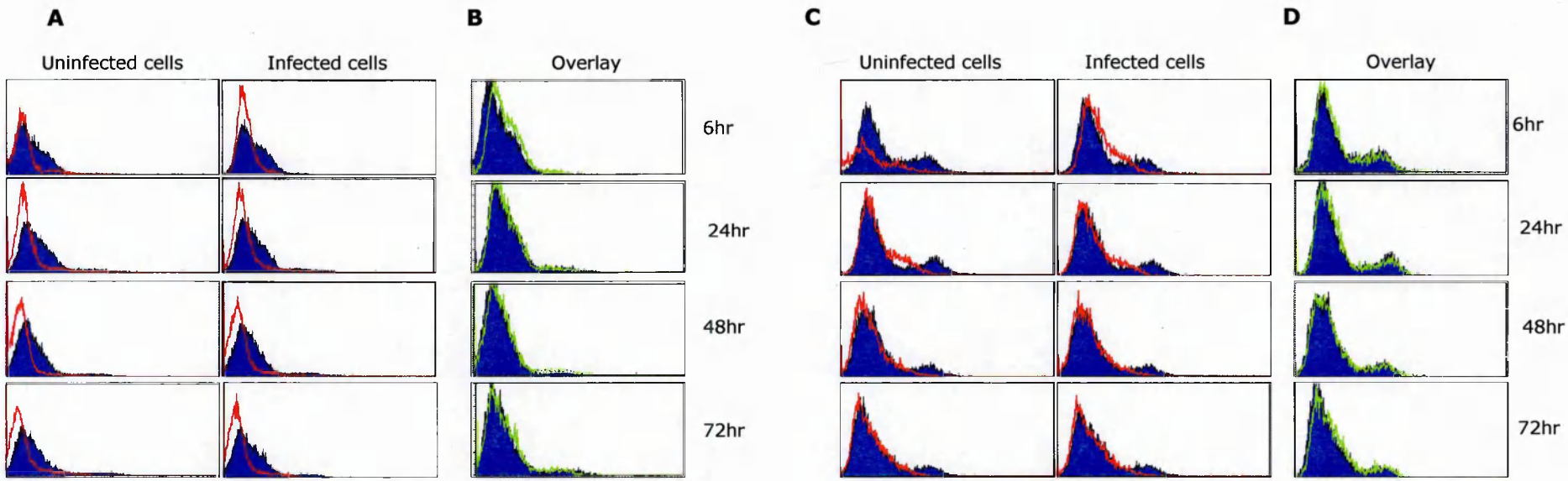
Figure 5.3 The expression of MHC class II (HLA DR) on uninfected lung epithelial cells. **(A)** HEp-2 cells **(B)** A549 cells. Key: **purple filed** - HLA DR; **red line** - isotype control.



E

Molecule	Cells	HEp-2				A549			
		6hr	24hr	48hr	72hr	6hr	24hr	48hr	72hr
HLA ABC	UNINFECTED	74.51	67.32	44.08	65.11	28.54	21.71	19.27	38.57
	INFECTED	69.78	59.46	45.6	59.98	28.24	24.72	20.68	29.65
	% CHANGE	-6.35	-11.68	3.45	-7.88	-1.05	13.86	7.32	-23.13

Figure 5.4 The expression of MHC class I (HLA ABC) on lung epithelial cells following *C. pneumoniae* infection. Cells were infected with *C. pneumoniae* (MOI=1) and samples were collected 6, 24, 48 and 72 hours after infection. **(A)** HEp-2 cells **(B)** HEp-2 cells overlay of infected and uninfected cells. **(C)** A549 cells **(D)** A549 cells overlay of infected and uninfected cells **(E)** MFI values of HLA ABC expression on A549 and HEp-2 cells and difference following infection. Key: **purple filed** - HLA ABC; **red line** isotype control. In the overlay: **purple filed** - uninfected cells: **green line** –infected cells.



E

Molecule	Cells	HEp-2 6hr	HEp-2 24hr	HEp-2 48hr	HEp-2 72hr	A549 6hr	A549 24hr	A549 48hr	A549 72hr
CD54	UNINFECTED	5.04	5.09	3.98	5.18	11.78	13.15	8.26	7.95
	INFECTED	4.89	4.63	4.28	4.86	10.49	11.83	8.32	9.2
	% CHANGE	-2.98	-9.04	7.54	-6.18	-10.95	-10.04	0.73	15.72

Figure 5.5 The expression of CD54 on lung epithelial cells following *C. pneumoniae* infection. Cells were infected with *C. pneumoniae* (MOI=1) and samples were collected 6, 24, 48 and 72 hours after infection. **(A)** HEp-2 cells **(B)** HEp-2 cells overlay of infected and uninfected cells. **(C)** A549 cells **(D)** A549 cells overlay of infected and uninfected cells **(E)** MFI values of HLA ABC expression on A549 and HEp-2 cells and difference following infection. Key for A and C: **purple filed** - CD54; **red line**- isotype control. In the overlays: **purple filed** - uninfected cells; **green line** –infected cells.

In summary, *C. pneumoniae* infection does not affect the expression of MHC class I molecules on lung epithelial cells (A549 and HEp-2), and its effects of CD54 expression are unclear, but as far as this study is concerned *C. pneumoniae* does not affect CD54 expression either. The next step was to analyse what effect chlamydial infection has on MdMs.

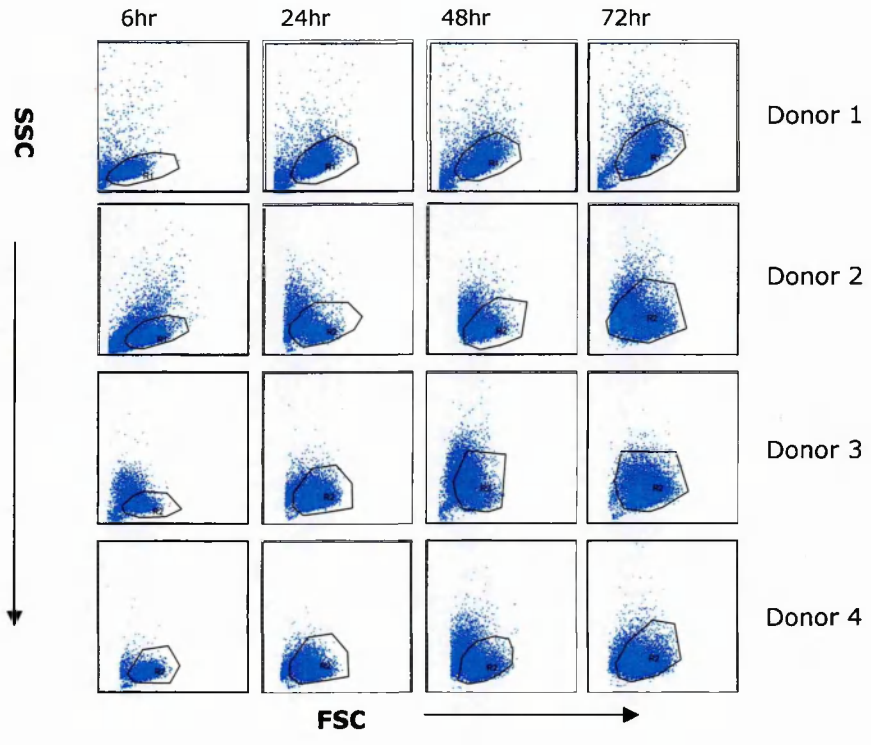
5.3.2 *C. pneumoniae* infection of MdMs modulates expression of surface molecules

Expression of CD14, CD40, CD45, CD54, CD91, HLA ABC, HLADR and TLR4 was analysed by flow cytometry on MdMs from 4 donors and the results are presented in full as histograms grouped by target molecules, and in Tables of corresponding MFI values and percentage of expression change (Figure 5.7 to 5.14). Forward scatter vs side scatter profiles of MdMs from the donors are presented in Figure 5.6. From these it is clear that the cells have similar profiles and that the gating is similar.

All the molecules were expressed on the MdMs analysed. *C. pneumoniae* infection modulated the expression in different ways. CD14 was up-regulated in two donors with small changes in the other two (Figure 5.7). CD40 was up-regulated in two, down-regulated, with no changes in the one donor (Figure 5.8) CD45 was also up-regulated in two donors and down-regulated in the other two; however the changes were smaller (Figure 5.9). The expression of CD54 was up-regulated in 2 and down-regulated in the other two donors (Figure 5.10). The effect of infection on CD91 was variable with up-regulation early in the infection and down-regulation later in two donors, and a mixed effect on the other two donors (Figure 5.11). MHC class I and II were both down-regulated in all four donors (Figures 5.12 and 5.13).

The expression of TLR4 was very low in all donors and the infection had a variable effect on TLR4 expression.

A



B

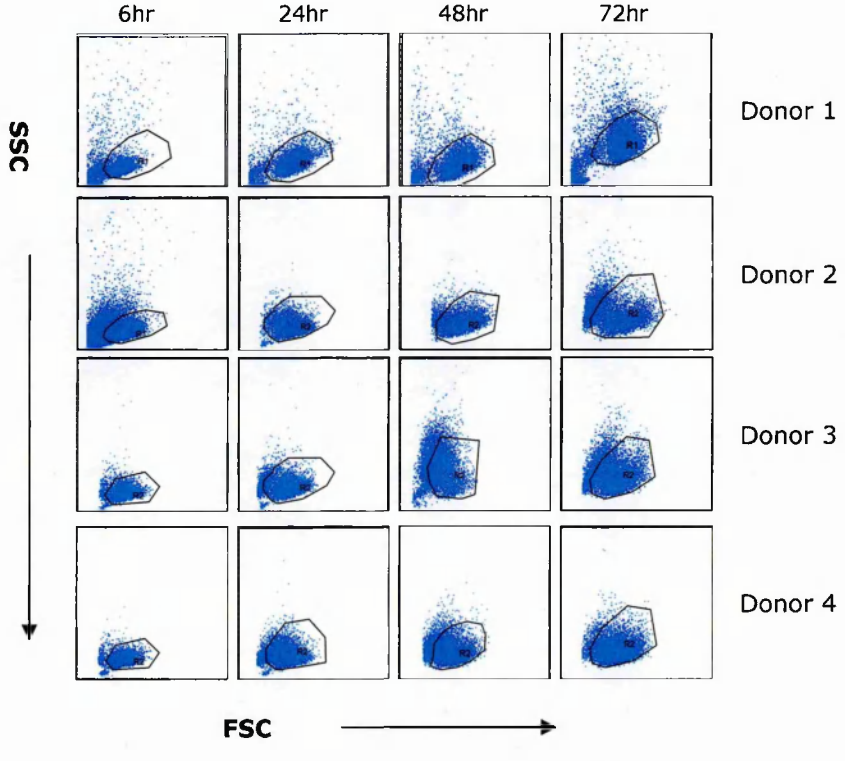
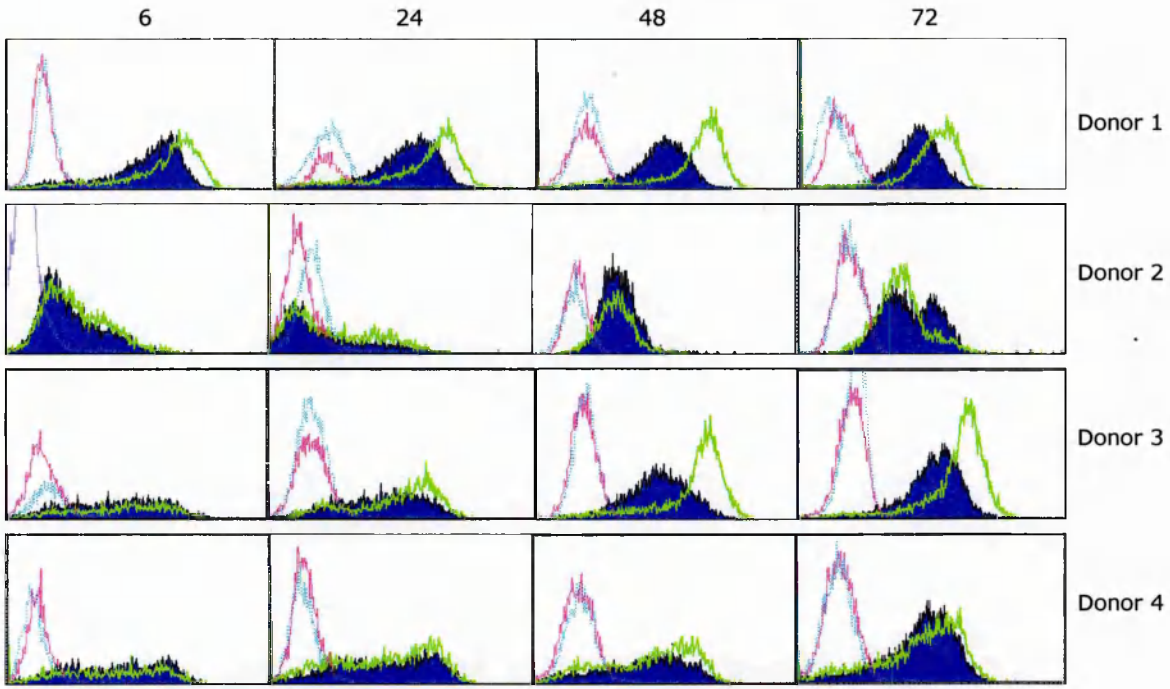


Figure 5.6 Forward scatter Vs side scatter (FSC/SSC) profiles of MdMs from 4 donors. **(A)** uninfected cells **(B)** infected cells. Key: X axis –FSC; Y axis - SSC.

A

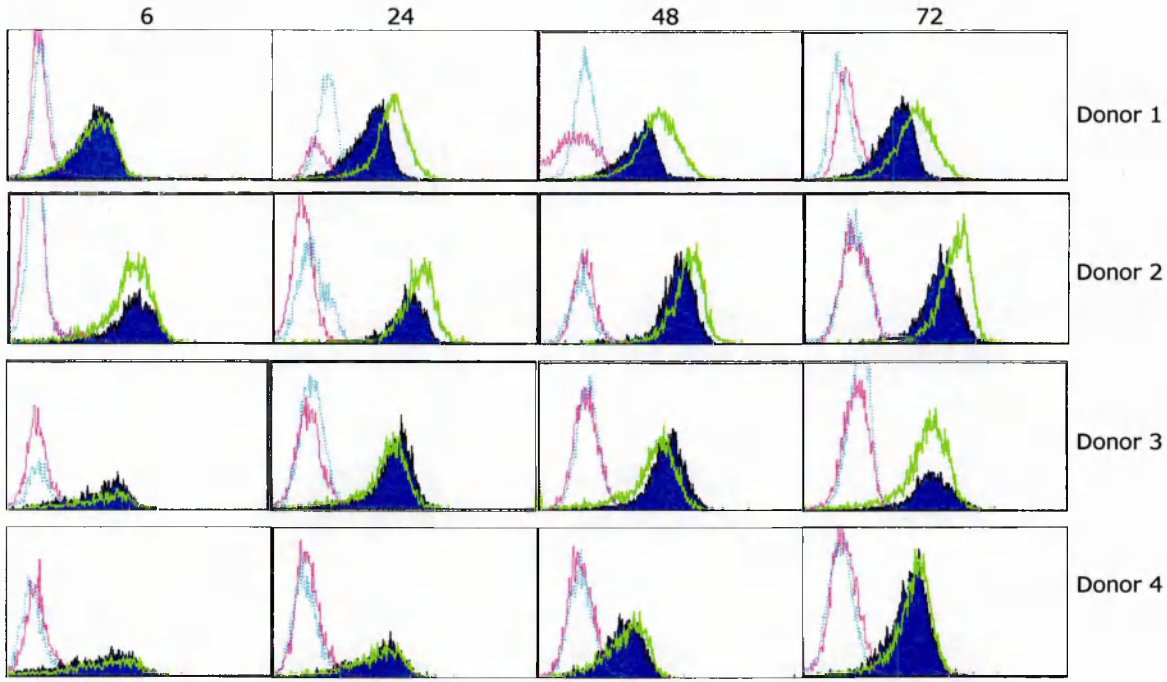


B

Donors		CD14			
		6hr	24hr	48hr	72hr
1	UNINFECTED	186.26	118.22	88.79	68.34
	INFECTED	374.01	288.98	300.23	120.32
	% CHANGE	**100.80**	**144.44**	**283.13**	**76.06**
2	UNINFECTED	14.69	14.86	37.66	41.94
	INFECTED	18.00	20.54	38.41	79.26
	% CHANGE	**22.53**	**38.22**	**1.99**	**88.98**
3	UNINFECTED	94.59	78.78	108.60	134.08
	INFECTED	118.44	291.99	311.60	338.20
	% CHANGE	**25.21**	**270.64**	**186.92**	**152.24**
4	UNINFECTED	105.59	128.39	138.39	107.73
	INFECTED	95.90	130.05	167.62	128.49
	% CHANGE	**-9.18**	**1.29**	**21.12**	**19.27**

Figure 5.7 Expression of CD14 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD14 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

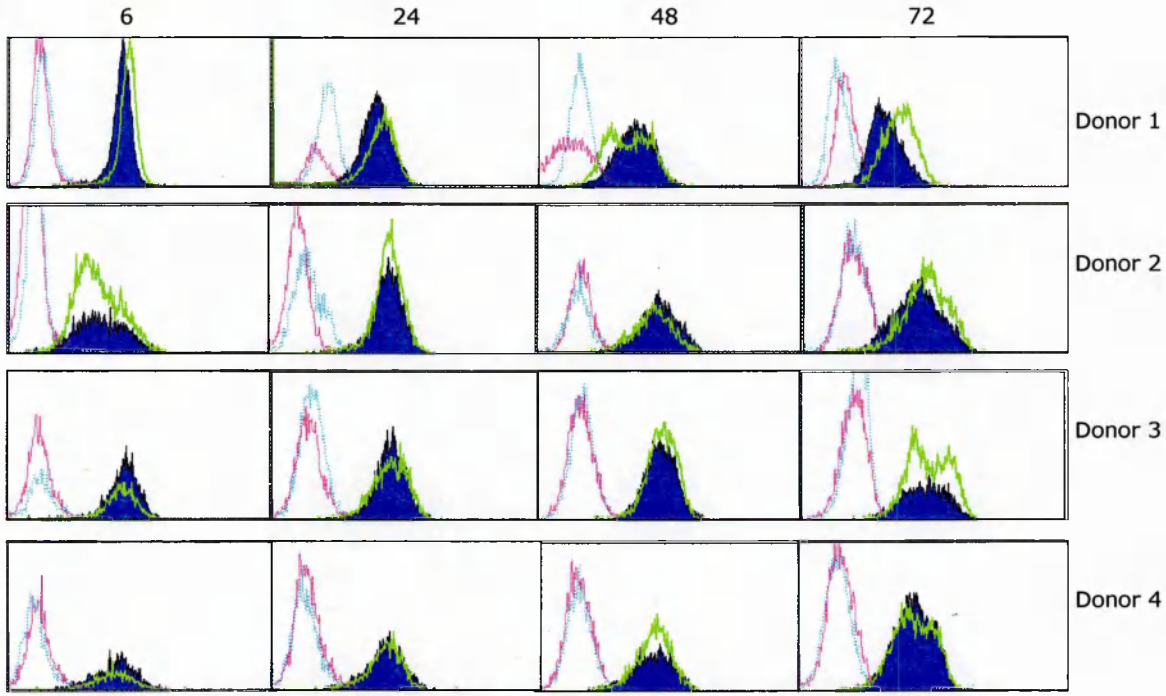


B

Donor		CD40 6hr	CD40 24hr	CD40 48hr	CD40 72hr
1	UNINFECTED	23.72	33.85	37.73	28.17
	INFECTED	23.62	70.41	76.23	56.62
	% CHANGE	**-0.42**	**108.01**	**102.04**	**100.99**
2	UNINFECTED	89.53	118.76	135.25	127.84
	INFECTED	78.71	173.64	185.20	208.15
	% CHANGE	**-12.09**	**46.21**	**36.93**	**62.82**
3	UNINFECTED	37.60	79.86	91.76	105.76
	INFECTED	36.30	59.41	64.25	96.06
	% CHANGE	**-3.46**	**-25.61**	**-29.98**	**-9.17**
4	UNINFECTED	37.16	45.44	23.57	46.37
	INFECTED	46.38	45.79	24.18	52.70
	% CHANGE	**24.81**	**0.77**	**2.59**	**13.65**

Figure 5.8 Expression of CD40 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD40 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

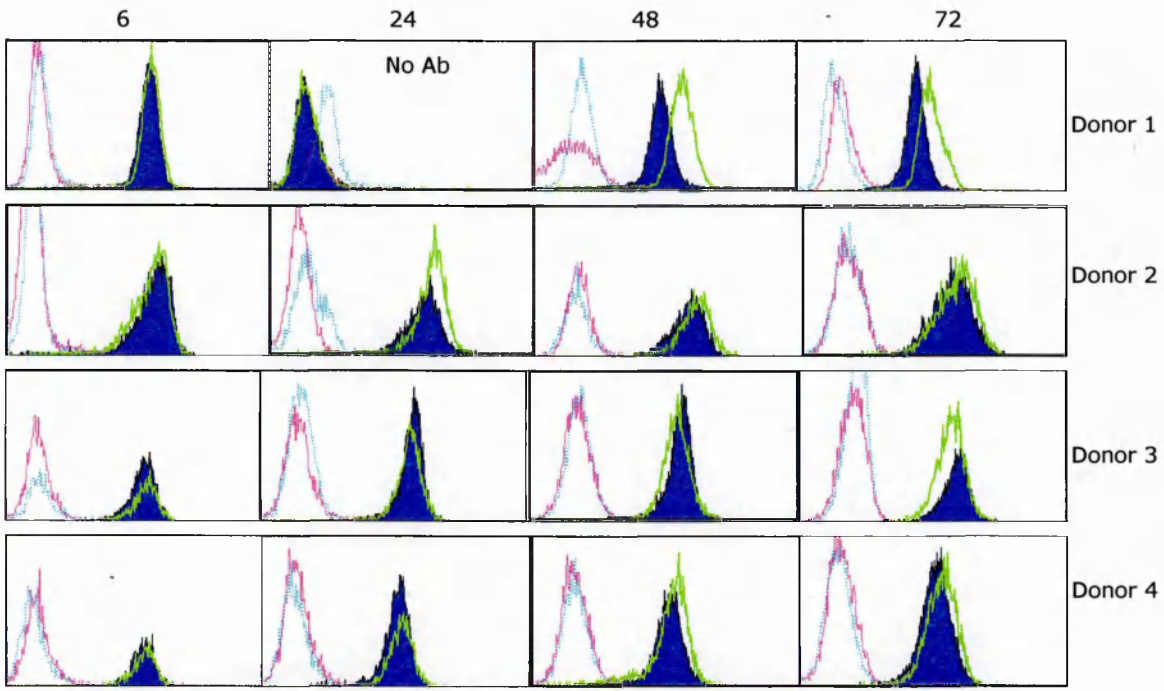


B

Donors		CD45			
		6hr	24hr	48hr	72hr
1	UNINFECTED	50.56	40.39	38.52	20.29
	INFECTED	62.40	43.56	33.13	32.58
	% CHANGE	**23.42**	**7.85**	**-13.99**	**60.57**
2	UNINFECTED	32.31	66.56	81.97	67.92
	INFECTED	28.64	67.83	65.38	88.97
	% CHANGE	**-11.36**	**1.91**	**-20.24**	**30.99**
3	UNINFECTED	60.54	68.17	83.93	88.73
	INFECTED	52.86	69.52	92.02	109.09
	% CHANGE	**-12.69**	**1.98**	**9.64**	**22.95**
4	UNINFECTED	45.67	55.06	64.67	59.98
	INFECTED	42.34	53.03	63.25	60.21
	% CHANGE	**-7.29**	**-3.69**	**-2.20**	**0.38**

Figure 5.9 Expression of CD45 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD45 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs with the percentage of change. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

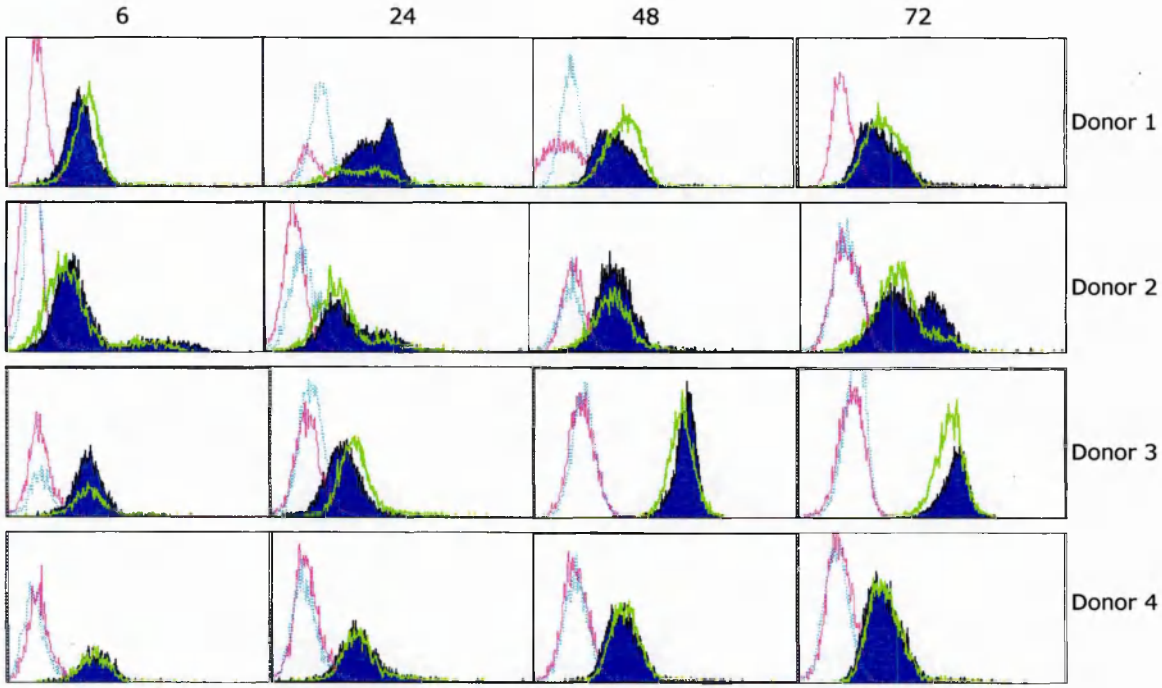


B

Donors		CD54 6hr	24hr	48hr	72hr
1	UNINFECTED	134.27	N/A	77.01	60.20
	INFECTED	144.79	181.55	156.21	103.76
	% CHANGE	**7.83**	**N/A**	**102.84**	**72.36**
2	UNINFECTED	173.43	214.46	236.01	227.95
	INFECTED	156.39	277.47	265.79	270.26
	% CHANGE	**-9.83**	**29.38**	**-12.86**	**-9.68**
3	UNINFECTED	116.63	194.81	182.71	237.81
	INFECTED	120.99	179.59	159.22	214.87
	% CHANGE	**3.74**	**-7.81**	**-12.86**	**-9.65**
4	UNINFECTED	114.29	114.30	119.04	118.72
	INFECTED	119.86	124.82	131.03	141.24
	% CHANGE	**4.87**	**9.20**	**10.07**	**18.97**

Figure 5.10 Expression of CD54 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD54 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells. For donor 1 at 24 hours, the antibody was not added by mistake.

A

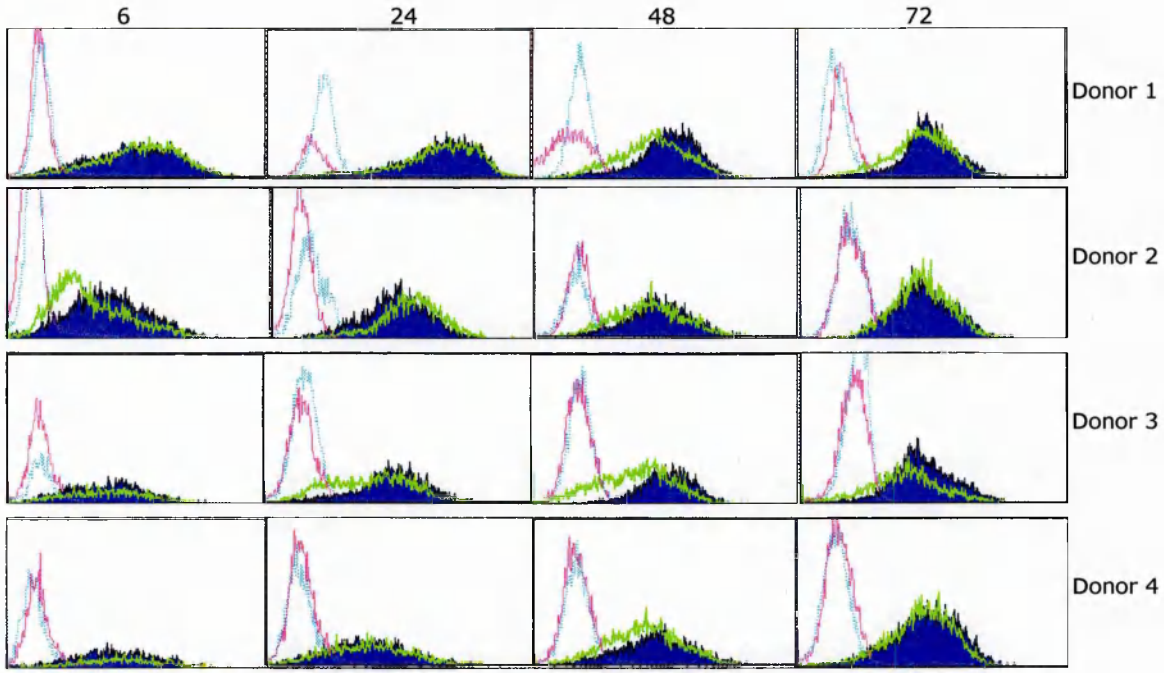


B

Donors		CD91 6hr	24hr	48hr	72hr
1	UNINFECTED	19.77	57.51	72.58	37.26
	INFECTED	26.92	102.47	61.85	21.33
	% CHANGE	**36.17**	**78.18**	**-14.78**	**-42.75**
2	UNINFECTED	41.79	34.31	22.32	72.17
	INFECTED	20.50	24.76	19.22	52.76
	% CHANGE	**-50.95**	**-27.83**	**-13.89**	**-26.89**
3	UNINFECTED	17.97	15.59	44.54	36.06
	INFECTED	22.31	24.03	22.40	36.76
	% CHANGE	**24.15**	**54.14**	**-49.71**	**1.94**
4	UNINFECTED	25.34	27.52	26.44	26.87
	INFECTED	27.15	25.82	26.44	26.24
	% CHANGE	**7.14**	**-6.18**	**0.00**	**-2.34**

Figure 5.11 Expression of CD91 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD91 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

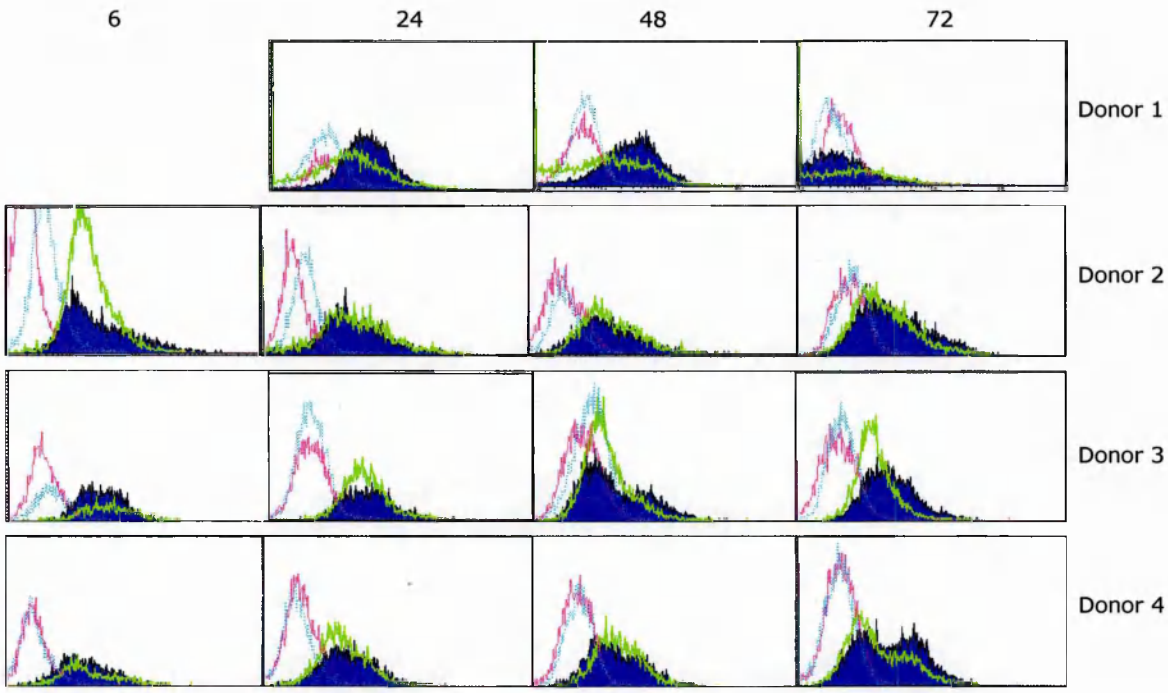


B

Donors		HLA DR 6hr	24hr	48hr	72hr
1	UNINFECTED	155.48	609.76	154.45	129.87
	INFECTED	166.50	620.19	99.08	94.61
	% CHANGE	**-65.19**	**1.70**	**-27.15**	**-35.85**
2	UNINFECTED	54.12	108.50	105.43	103.50
	INFECTED	34.14	173.27	79.70	93.86
	% CHANGE	**-79.50**	**59.70**	**-9.31**	**-24.90**
3	UNINFECTED	51.76	123.17	132.86	103.79
	INFECTED	51.79	82.68	60.80	64.42
	% CHANGE	**0.06**	**-32.87**	**-37.93**	**-54.24**
4	UNINFECTED	74.86	58.23	107.75	124.01
	INFECTED	85.00	58.84	72.46	101.62
	% CHANGE	**13.55**	**1.05**	**-18.05**	**-37.75**

Figure 5.12 Expression of HLA DR by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for HLA DR expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

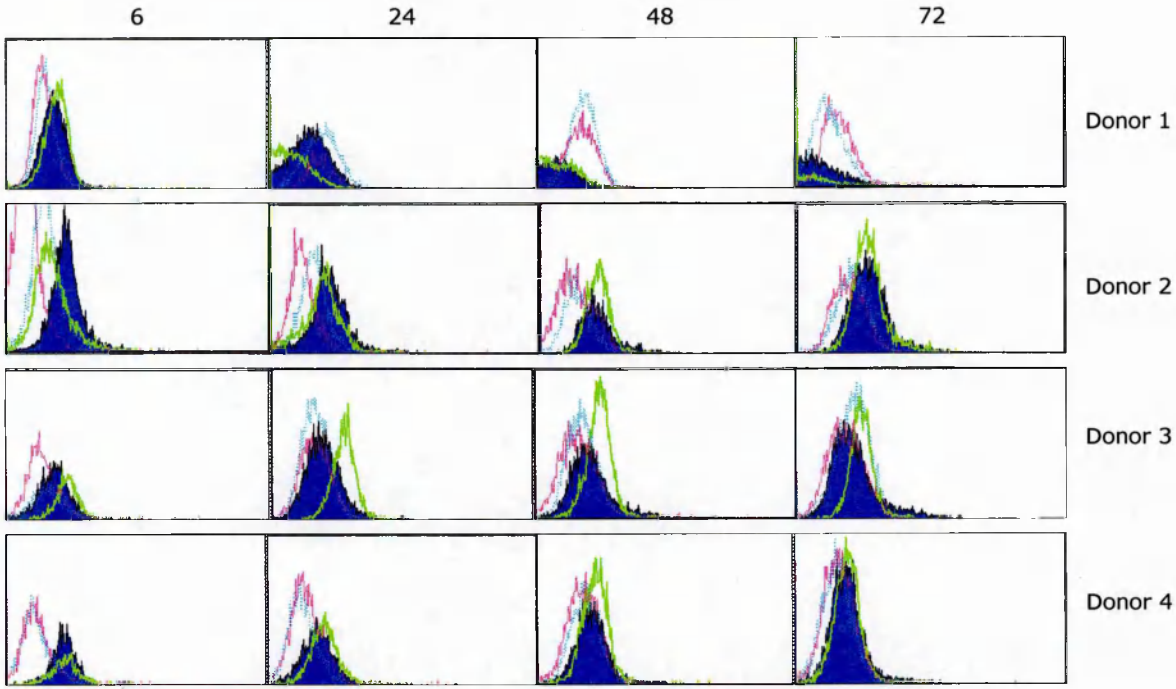


B

Donors		HLA ABC 6hr	24hr	48hr	72hr
1	UNINFECTED	N/A	34.78	38.36	4.64
	INFECTED	N/A	26.07	20.74	5.63
	% CHANGE	**N/A**	**-25.04**	**-45.93**	**21.34**
2	UNINFECTED	41.07	38.84	22.35	46.02
	INFECTED	21.75	45.06	22.86	37.34
	% CHANGE	**-47.07**	**16.01**	**2.28**	**-18.86**
3	UNINFECTED	31.72	37.72	24.59	26.48
	INFECTED	40.01	27.75	16.79	16.17
	% CHANGE	**26.13**	**-26.43**	**-31.72**	**-38.94**
4	UNINFECTED	19.87	25.54	24.57	36.22
	INFECTED	24.32	18.64	23.42	21.94
	% CHANGE	**22.40**	**-27.02**	**-4.68**	**-39.43**

Figure 5.13 Expression of HLA ABC by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for HLA ABC expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells. The missing quadrant for donor 1 at 6 hours – the sample was destroyed during the experiment.

A



B

Donors		TLR4 6hr	24hr	48hr	72hr
1	UNINFECTED	5.79	4.79	1.79	1.90
	INFECTED	6.30	2.10	1.91	1.20
	% CHANGE	**8.81**	**-56.16**	**-6.70**	**-36.84**
2	UNINFECTED	8.51	8.66	8.42	13.38
	INFECTED	5.51	6.71	7.52	12.79
	% CHANGE	**-35.25**	**-22.52**	**-10.69**	**-4.41**
3	UNINFECTED	5.68	6.72	6.35	9.15
	INFECTED	9.16	12.71	9.12	9.74
	% CHANGE	**61.27**	**89.14**	**43.62**	**6.45**
4	UNINFECTED	7.42	6.56	6.69	6.54
	INFECTED	7.83	7.07	7.25	6.16
	% CHANGE	**5.53**	**7.77**	**8.37**	**-5.81**

Figure 5.14 Expression of TLR4 by MdMs following *C. pneumoniae* infection. Cells were maintained in culture for 48 hours after isolation and then infected with *C. pneumoniae* (MOI=1). Samples were collected 6, 24, 48 and 72 hours after infection and analysed for TLR4 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

In summary, *C. pneumoniae* infection of MdMs modulates the expression of surface molecules on MdMs; however there are differences between donors in the baseline levels of expression of different molecules and also in how *C. pneumoniae* infection affects their expression.

To obtain a more complete picture as to how MdMs are activated as part of the immune response to *C. pneumoniae* infections, and how they interact with infected ling epithelial cells, expression of the same surface molecules was further analysed following activation of MdMs by medium derived from infected epithelial cells.

5.3.3 Conditioned medium modulates the expression of cell-surface molecules on MdMs

MdMs were treated with conditioned medium (CM) and the change in expression of surface molecules was analysed in the same way as described above. The forward scatter vs side scatter profiles of each of the donors are presented in Figure 5.15. The results are presented as in the previous section with histograms for each individual donor and a table of corresponding MFI values in Figures 5.16 to 5.23.

Overall, the effect of CM-treatment on MdMs was less pronounced than *C. pneumoniae* infection, and variability between donors is greater. There was a mixed effect on the expression of CD40, CD45 and TLR4 (Figures 5.17, 5.18 and 5.23). CD14 and CD54 were down-regulated in two donors and up-regulated in one (Figures 5.16 and 5.19) and the effect on CD91, MHC class I and II was the opposite, with up-regulation in two donors and down-regulation in one (Figures 5.20, 5.21 and 5.22).

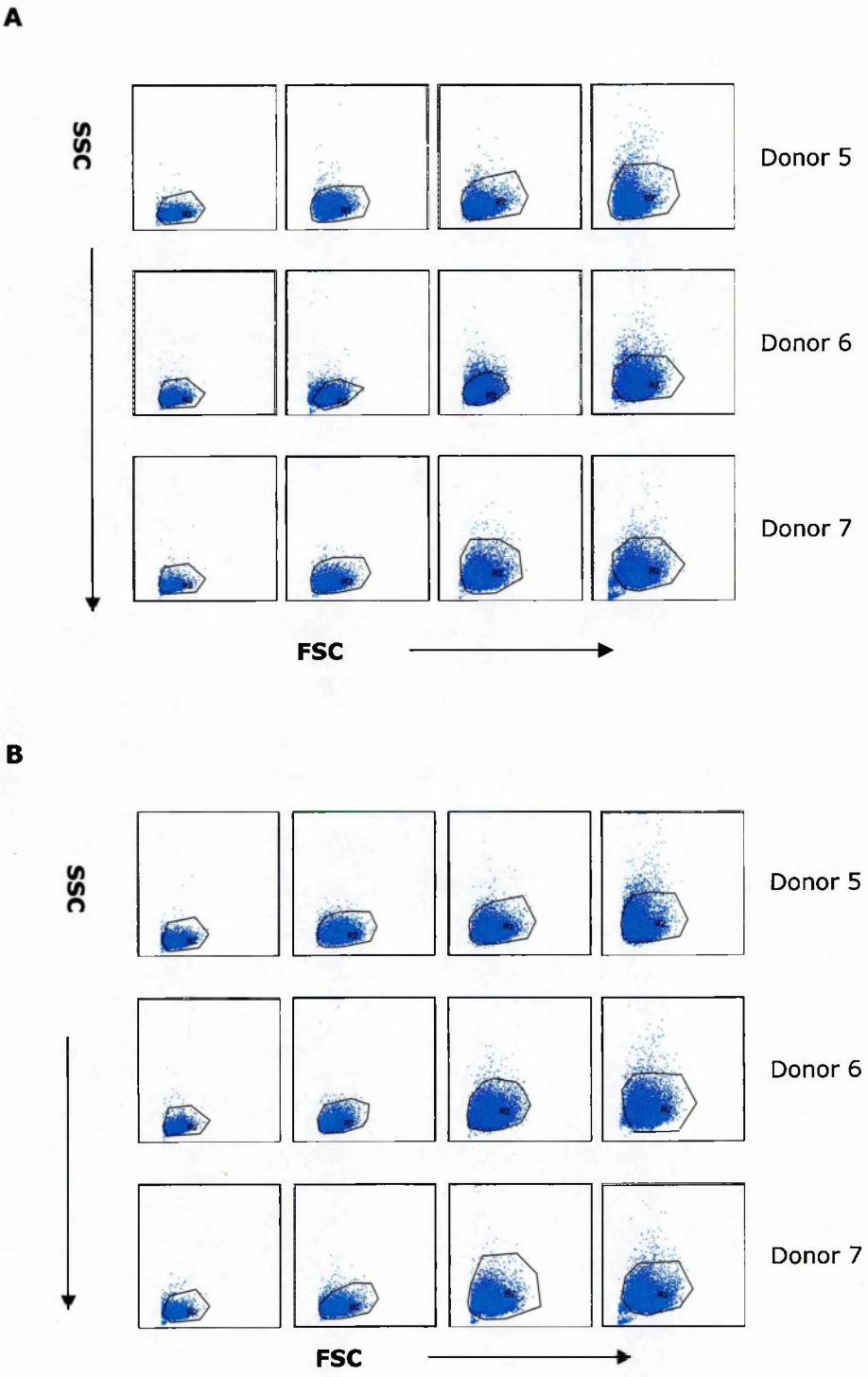
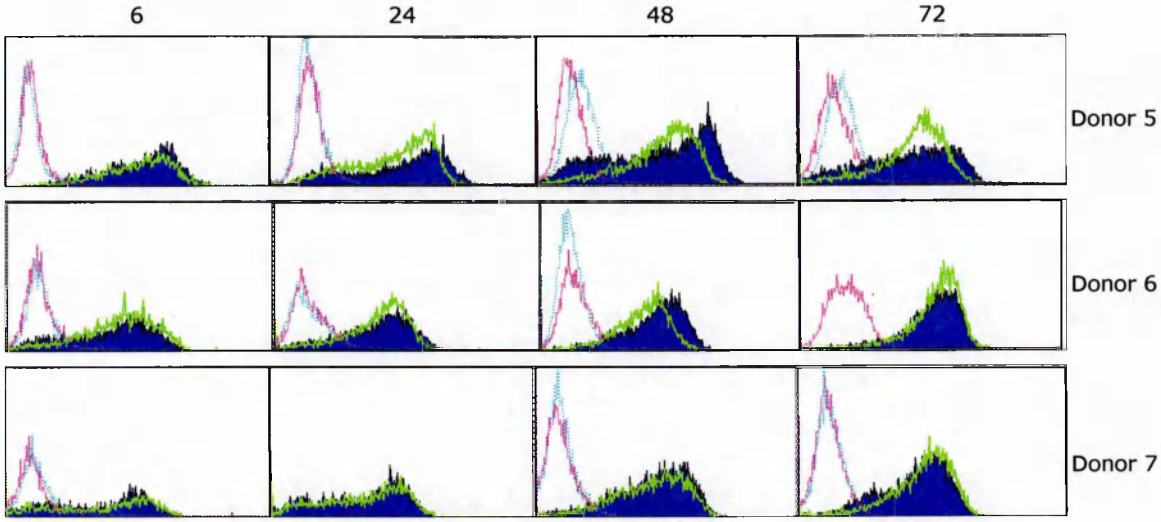


Figure 5.15 Forward scatter Vs side scatter (FSC/SSC) profiles of MdMs from 3 donors. **(A)** uninfected cells **(B)** infected cells. Key: X axis –FSC; Y axis SSC.

A

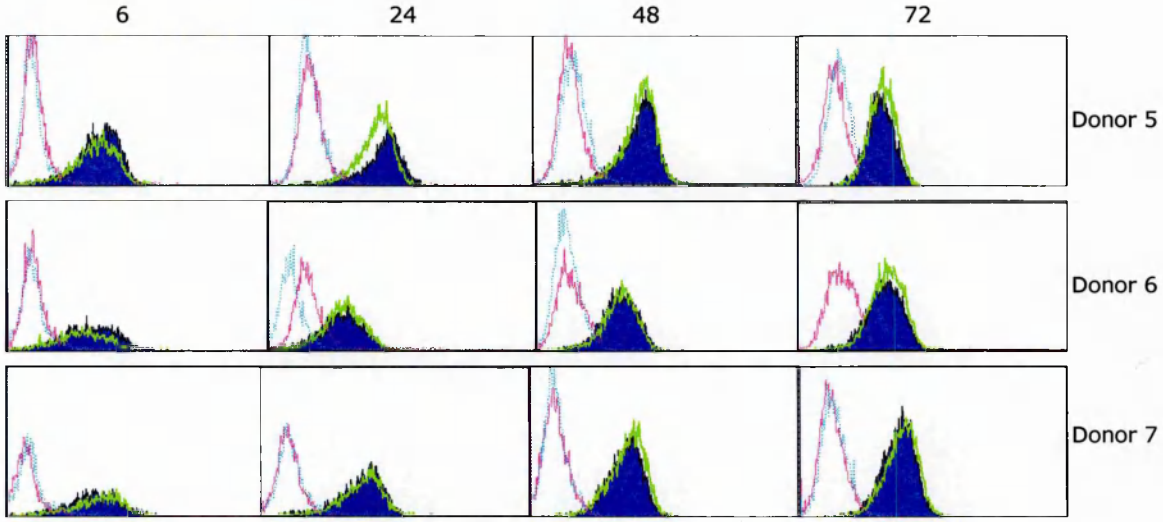


B

Donors		CD14 6hr	CD14 24hr	CD14 48hr	CD14 72hr
5	UNTREATED	140.02	160.51	133.88	92.24
	TREATED	132.51	135.69	107.28	79.34
	% CHANGE	**-5.36**	**-15.46**	**-19.87**	**-13.99**
6	UNTREATED	82.06	59.42	71.89	63.12
	TREATED	75.33	73.78	44.31	67.87
	% CHANGE	**-8.20**	**24.17**	**-38.36**	**7.53**
7	UNTREATED	50.52	43.88	88.08	89.01
	TREATED	64.65	46.71	90.32	102.43
	% CHANGE	**27.97**	**6.45**	**2.54**	**15.08**

Figure 5.16 Expression of CD14 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD14 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

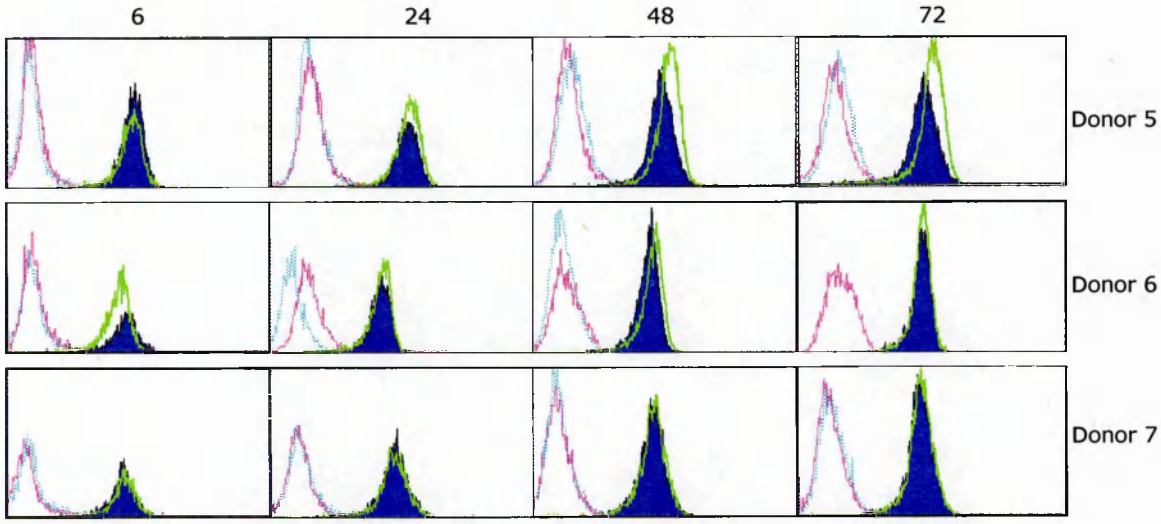


B

Donors		CD40 6hr	24hr	48hr	72hr
5	UNTREATED	26.97	53.20	41.77	17.66
	TREATED	23.85	41.11	41.40	19.05
	% CHANGE	**-11.57**	**-22.73**	**-0.89**	**7.87**
6	UNTREATED	23.59	14.55	19.27	22.11
	TREATED	17.55	16.17	20.20	23.69
	% CHANGE	**-25.60**	**11.13**	**4.83**	**7.15**
7	UNTREATED	24.94	39.23	28.99	70.41
	TREATED	26.47	37.52	31.09	36.63
	% CHANGE	**6.13**	**-4.36**	**7.24**	**-47.98**

Figure 5.17 Expression of CD40 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD40 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

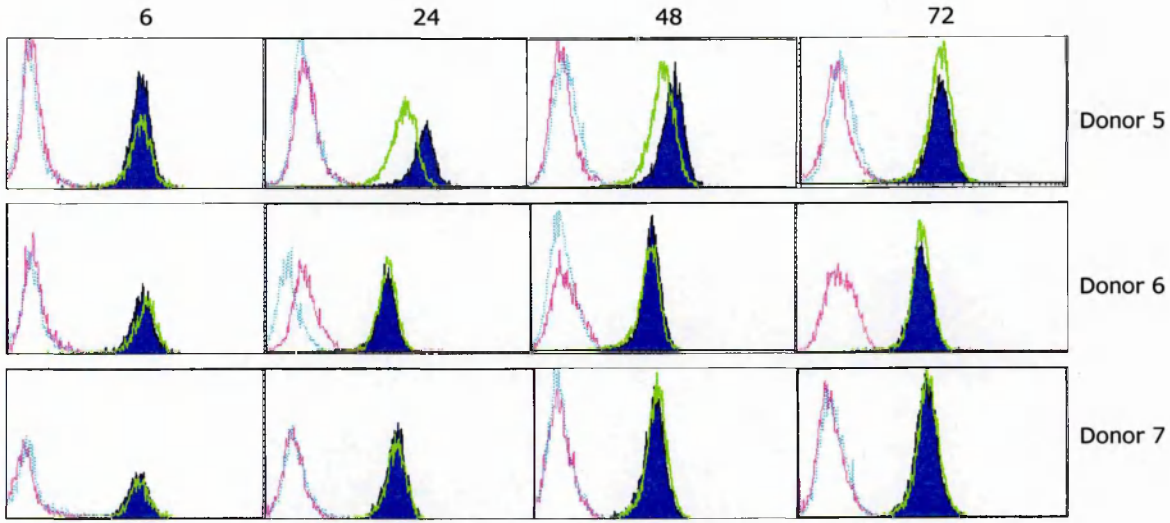


B

Donors		CD45 6hr	24hr	48hr	72hr
5	UNTREATED	77.68	108.80	84.68	77.59
	TREATED	71.67	115.09	107.26	102.33
	% CHANGE	**-7.74**	**5.78**	**26.67**	**31.89**
6	UNTREATED	60.14	43.74	51.43	71.74
	TREATED	46.15	43.07	60.55	71.71
	% CHANGE	**-23.26**	**-1.53**	**17.73**	**-0.04**
7	UNTREATED	53.66	73.44	59.99	71.11
	TREATED	55.69	71.02	61.54	70.80
	% CHANGE	**3.78**	**-3.30**	**0.93**	**-0.44**

Figure 5.18 Expression of CD45 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD45 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

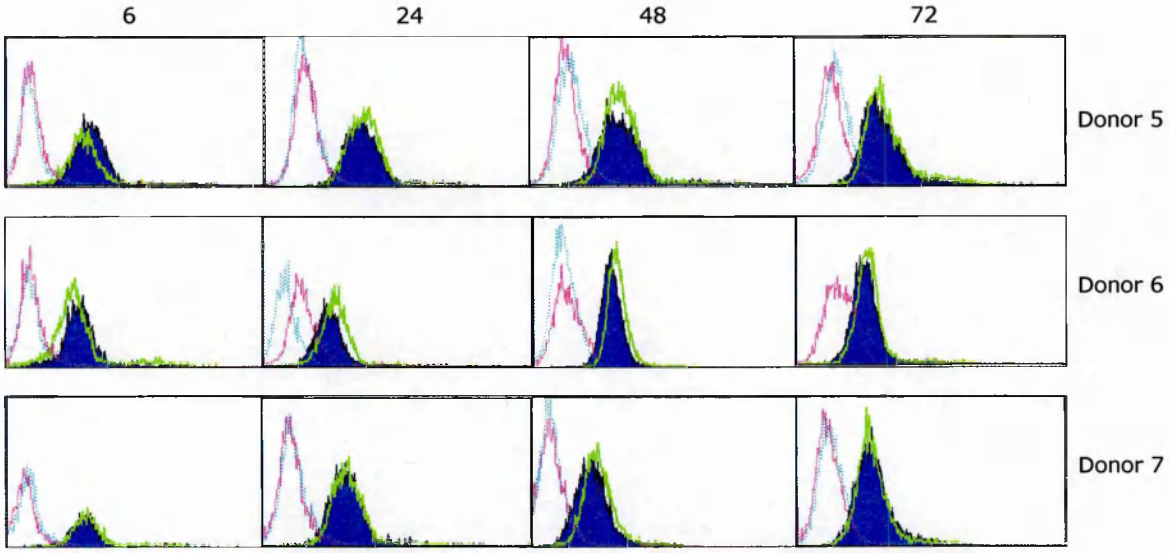


B

Donors		CD54 6hr	CD54 24hr	CD54 48hr	CD54 72hr
5	UNTREATED	99.57	229.83	153.68	129.02
	TREATED	97.62	116.27	107.19	128.98
	% CHANGE	**-1.96**	**-49.41**	**-30.25**	**-0.03**
6	UNTREATED	192.60	61.80	59.22	70.22
	TREATED	110.65	63.82	55.42	71.74
	% CHANGE	**-42.55**	**3.27**	**-6.42**	**2.16**
7	UNTREATED	89.85	88.31	60.31	85.17
	TREATED	91.86	87.09	64.39	89.25
	% CHANGE	**2.24**	**-1.38**	**6.77**	**4.79**

Figure 5.19 Expression of CD54 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD54 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

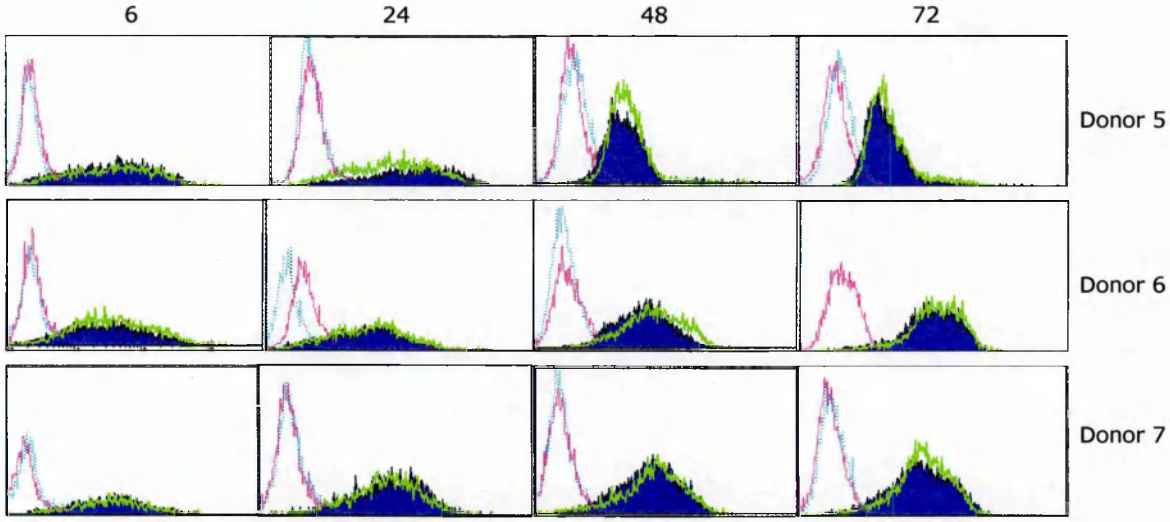


B

Donors		CD91 6hr	24hr	48hr	72hr
5	UNTREATED	21.80	33.60	28.52	25.64
	TREATED	20.35	31.52	30.33	30.44
	% CHANGE	**-6.65**	**-6.19**	**6.35**	**18.72**
6	UNTREATED	15.76	13.74	14.50	12.12
	TREATED	21.53	13.12	18.07	14.71
	% CHANGE	**36.61**	**-4.51**	**24.62**	**21.37**
7	UNTREATED	29.11	24.83	10.73	16.28
	TREATED	26.41	21.40	14.68	15.34
	% CHANGE	**-9.28**	**-13.81**	**36.81**	**-5.77**

Figure 5.20 Expression of CD91 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for CD91 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

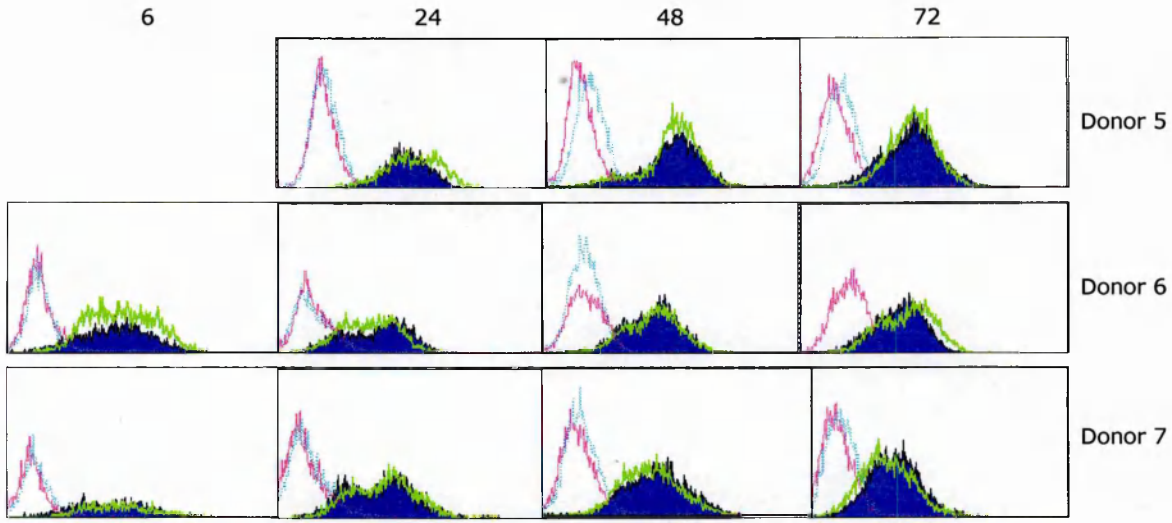


B

Donors		HLA DR 6hr	24hr	48hr	72hr
5	UNTREATED	74.46	226.05	114.11	94.52
	TREATED	76.51	121.63	195.71	195.44
	% CHANGE	**2.75**	**-46.19**	**106.77**	**71.51**
6	UNTREATED	61.93	52.94	55.25	127.60
	TREATED	69.56	67.04	74.96	141.67
	% CHANGE	**12.32**	**26.63**	**11.03**	**35.67**
7	UNTREATED	54.06	136.17	72.98	98.33
	TREATED	73.25	100.75	81.11	98.16
	% CHANGE	**35.50**	**-26.01**	**-0.17**	**11.14**

Figure 5.21 Expression of HLA DR by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for HLA DR expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

A

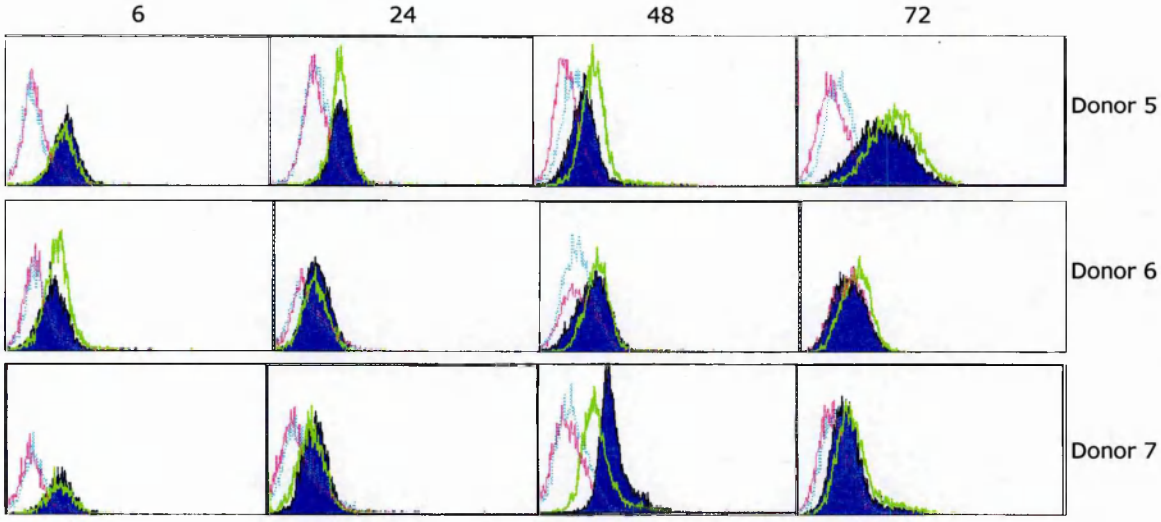


B

Donors		HLA ABC 6hr	24hr	48hr	72hr
5	UNTREATED	N/A	99.63	106.36	64.55
	TREATED	N/A	140.77	107.85	60.95
	% CHANGE	**N/A**	**41.29**	**1.40**	**-5.58**
6	UNTREATED	64.98	43.10	55.56	38.87
	TREATED	77.47	31.36	60.22	53.86
	% CHANGE	**19.22**	**-27.24**	**8.39**	**38.56**
7	UNTREATED	55.22	58.58	74.07	34.44
	TREATED	77.29	63.81	48.91	27.48
	% CHANGE	**39.97**	**8.93**	**-33.97**	**-20.21**

Figure 5.22 Expression of HLA ABC by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for HLA ABC expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells. The 6 hour sample for donor 5 was destroyed during the experiment.

A



B

Donors		TLR4 6hr	TLR4 24hr	TLR4 48hr	TLR4 72hr
5	UNTREATED	8.53	11.63	6.39	24.50
	TREATED	7.36	11.36	8.88	35.22
	% CHANGE	**-13.72**	**-2.32**	**38.97**	**43.76**
6	UNTREATED	5.85	4.59	7.88	5.94
	TREATED	6.51	4.22	6.96	7.45
	% CHANGE	**11.28**	**-8.06**	**-11.68**	**25.42**
7	UNTREATED	6.62	5.49	16.61	6.89
	TREATED	6.48	4.59	9.56	9.34
	% CHANGE	**-2.11**	**-16.39**	**-42.44**	**35.56**

Figure 5.23 Expression of TLR4 by MdMs treated with conditioned medium. Cells were maintained in culture for 48 hours after isolation and then treated with 10% conditioned medium. Samples were collected 6, 24, 48 and 72 hours after infection and analysed for TLR4 expression by flow cytometry. **(A)** histograms **(B)** table of corresponding MFIs. Key: **purple field** – uninfected cells; **green line** -infected cells; **blue line**- isotype control for uninfected cells; **red line**- isotype control for infected cells.

To summarise, the expression of cell-surface molecules is modulated by *C. pneumoniae* either by infection or by the inflammatory response that is induced in infected epithelial cells.

5.4 ***Discussion***

Cell to cell communication is a central component of building an immune response. This communication is largely dependent on the activation of cell-surface molecules (receptors) that transfer signals into the cell thereby activating it to respond. Certain pathogens, and intracellular pathogens in particular, such as cytomegalovirus or adenovirus, have devised mechanisms to modulate the expression of surface molecules (MHC class I down-regulation) as part of evading the host immune response (Benz et al., 2001, Slater and Campbell, 1997, Hou et al., 2002, Sparer and Gooding, 1998, Korner and Burgert, 1994, Bosse and Ades, 1991). The experiments described in this chapter were designed to investigate if *C. pneumoniae* modulates expression of surface molecules on both lung epithelial cells and MdMs.

Lung epithelial cells are the primary target of *C. pneumoniae* infection and they are activated to release pro-inflammatory modulators IL-6 and IL-8 (discussed in chapter 4). A selection of 4 cell-surface molecules with different functions in the immune response was chosen for analysis: classical MHC class I (HLA ABC), MHC class II (HLA DR); IFN-γ receptor α-chain (CD119) and CD54. Both A549 and HEp-2 cells express IFN-γ receptor in the resting state (Figure 5.2) and the expression levels are not changed by *C. pneumoniae* infection; however this is discussed in detail in chapter 6 in the context of chlamydial control by IFN-γ.

Neither A549 nor HEp-2 cell line express MHC class II (HLA DR; Figure 5.3), a finding that confirms a previous report by Gao et al., (1999) that A549 cells do not express MHC class II molecules in the resting state. MHC class II expression can be induced by inflammatory cytokines and some pathogens (Cunningham et al., 1994, Gao et al., 1999); however *C. pneumoniae* infection did not induce any expression.

Both cell lines express MHC class I (HLA ABC; Figure 5.4) and this was not modulated by chlamydial infection. The lack of effect on MHC class I expression does not necessarily mean that *C. pneumoniae* does not influence the MHC class I antigen processing and presentation pathway. It may change the rate of MHC class I recycling or in other ways affect antigen presentation by lung epithelial cells, and this can be addressed in future studies that could include functional analysis.

There was no clear demonstration of CD54 expression on A549 and HEp-2 cells and expression was not modulated by *C. pneumoniae* infection. The staining for CD54 can be repeated in future analysis with a multiple step analysis that may give a clearer result.

In retrospect it would have been interesting to look at other molecules including CD40 and TLR4. CD40 is expressed on a variety of cells including epithelial cells, and plays an important role in the inflammatory processes (Schonbeck and Libby, 2001a, Cagnoni et al., 2004). TLR4 plays an important role in bacterial immune responses, and it would have been interesting to analyse whether *C. pneumoniae* affects TLR4 expression on epithelial cells. A larger number of molecules analysed would have given a more complete picture of the role of epithelial cells in anti-chlamydial immune responses.

In the previous chapter it was shown that MdMs infected with *C. pneumoniae* release IL-8, IL-6, IL-1β, TNF-α and IL-10, and in this chapter it was demonstrated that *C. pneumoniae* also modulates the expression of cell-surface markers (Figures 5.7 to 5.14). It was further shown here that CM derived from infected A549 epithelial cells modulates the expression of cell-surface molecules differently from infection (Figures 5.16 to 5.23).

Eight surface molecules (CD14, CD40, CD45, CD54, CD91, HLA ABC, HLADR, TLR4) were chosen to give an indication of how *C. pneumoniae* affects surface molecule

expression and to obtain a more complete picture of how anti-chlamydial immunity developed. Infected MdMs expressed increased levels of CD14, CD40, CD45 and CD54 in two donors with no effect on the other two (Figures 5.7, 5.8, 5.9 and 5.10) The levels of MHC class I and II (Figures 5.12 and 5.13) were decreased in all donors. The effect on CD91 was different between donors with no consistency (Figure 5.11) and this is also the case for TLR4 (Figure 5.14). CM-treatment of MdMs down-regulated CD14 and CD54 expression in 2 donors and up-regulated it in one (Figures 5.16 and 5.19); up-regulated CD91, MHC class I and II expression in two donors and down-regulated it in one (Figures 5.20, 5.21 and 5.22), and there was a mixed effect on the expression of CD40, CD45 and TLR4 (Figures 5.17, 5.18 and 5.23).

On the whole, CM-treatment of MdMs had a weaker effect on the expression of surface molecules than *C. pneumoniae* infection. It is likely that CM is a cocktail of pro-inflammatory cytokines such as IL-8 and IL-6, anti-inflammatory mediators such as PGE_2, and chlamydial components such as LPS, cHSP60 and Inc proteins released by the infected epithelial cells. Such a balanced mix could activate MdMs so that they promote a weak inflammatory response.

As discussed in the previous chapter, the purity of the MdMs was about 70% and the presence of other cell population may affect the results. This was true in cytokine analysis however in the analysis of cell surface markers, I only gated on CD14 positive cells and subsequently analysed the expression of the other markers. So, the cells presented are all CD14 positive.

The most pronounced change observed was in the expression of CD14. The up-regulation of CD14 following infection (Figure 5.7) confirms previous results obtained from a MonoMac6 human macrophage cell line (Heinemann et al., 1996). Enhanced expression of CD14 could increase the responsiveness of MdMs to *C. pneumoniae*. On the other hand CM-treatment of MdMs down-regulates CD14 expression. These findings suggest that the level of CD14 expression on the surface of MdMs may have a

bearing on the severity of Chlamydia-induced immune responses. In this respect it is Interesting to note that a polymorphism in the CD14 promoter gene (C(-260)-->T) is associated with a higher density of CD14 expression on the macrophage surface and also with a higher risk of myocardial infarction and atherosclerosis in individuals expressing both alleles (CD14 TT) (Hubacek et al., 1999). Additionally, Eng *et al* have recently shown that CD14 TT homozygotes suffer a significantly higher risk of *C. pneumoniae* infection (Eng et ai., 2003). Taking that into consideration one could suggest that donor 1 may be more susceptible to *C. pneumoniae* compared to donor 2 (Figure 5.7) since they express different levels of CD14, however this requires a different experimental approach and plan.

The expression of MHC class I and class II molecules changes in the opposite way to CD14 (Figures 5.12 and 5.13). They are down-regulated by infection and up-regulated by CM-treatment. Down-regulation of MHC class I and II combined with enhanced levels of CD14 may be a contributing factor to long term persistent infections by increasing the cells' susceptibility to infection and by interfering with the effective activation of adaptive immune response and the creation of protective immunity. Other pathogens have been shown to manipulate the expression of MHC molecules as part of immune evasion, so it is possible that Chlamydiae do the same (Tomescu et al., 2003).

Even though the expression of CD14 was modulated by both infection and CM-treatment, there was no clear demonstration of a change in TLR4 expression levels by either treatment (Figures 5.14 and 5.23). This is interesting considering that CD14 is a key component of the TLR4 signalling pathway, and It has also been shown that both chlamydial LPS- and cHSP60- mediated activation of host cells is CD14/TLR4 dependent (Kol et al., 2000, Sasu et al., 2001, Costa et al., 2002, Prebeck et al., 2003). The lack of correlation in the modulation of CD14 and TLR4 expression by *C. pneumoniae* suggests that the expression of TLR4 and CD14 may not be coordinated.

Another way that cHSP60 may potentially activate immune responses is through

CD91, a common heat shock protein receptor. cHSP60 has an important role in both immediate inflammatory responses following *C. pneumoniae* infection and long-term in the development of atherosclerosis. There is an abundance of both cHSP and human HSP present during *C. pneumoniae* infections (discussed in detail in section 1.5.4.2 of chapter 1). Given the potential importance of cHSPs in inflammatory responses following *C. pneumoniae* infections the working hypothesis was that CD91 contributes to the immune responses following infection and that its expression on MdMs is modulated by *C. pneumoniae.* The level of CD91 expression was not greatly affected by infection, but CM-treatment enhanced CD91 levels. This may be due to the presence of cHSP in the CM and may contribute to immune responses. To further establish whether CD91 is involved in anti-chlamydial immunity a number of experiments can be conducted. For example: blocking of CD91 function with an antibody, using confocal analysis to see whether the receptor is being internalised following infection; analysis of CD91 expression by Real Time RT-PCR for quantification and also kinetic studies. Although CD91 is recognised as a common heat shock protein receptor, there are few reports on its role in the immune response following infections. Stebbing et al., (2003) have shown that high CD91 expression on macrophages is associated with the maintenance of long-term nonprogression of AIDS in HIV +ve patients, possibly by maintaining CD8+ T cell responses.

The remaining molecules analysed (CD40, CD45 and CD54) were all modulated similarly. CD45 was chosen for analysis solely as a leukocyte common antigen; however the results show that both infection and CM-treatment modulate its expression (Figures 5.9 and 5.18). Infection enhanced the expression while CM-treatment had a mixed effect. CD45 is involved in a number of T cell processes and it can act both as a positive and a negative regulator of T cell activation. It has recently been shown that CD45 acts as a phosphatase for Janus kinases (JAK), thereby acting as a negative regulator of cytokine receptor signalling. It has also been shown that targeted disruption of the *cd45* gene leads to enhanced cytokine and interferon-receptor-mediated activation of Jaks and Stats (Irie-Sasaki et al., 2001, Penninger et

al., 2001, Irie-Sasaki et al., 2003). As I mentioned before, IFN-γ is the key cytokine in controlling chlamydial growth and *C. pneumoniae*-mediated up-regulation of CD45 may interfere with IFN-γ dependent control of chlamydial growth. Findings presented here indicated that CD45 may play a crucial role in the outcome of *C. pneumoniae* infection, however this remains to be analysed.

CD40 is expressed on a number of different types of both lymphoid and non-lymphoid cells, including macrophages, endothelial and epithelial cells (Schonbeck and Libby, 2001a, Cagnoni et al., 2004). It was originally thought that CD40 is involved only in T/B lymphocyte interactions, mediating T cell–dependent B-cell activation and differentiation required for humoral immune responses. However more recently it has become clear that CD40/CD40L is a key part of inflammation and it has been associated with pathogenic processes of chronic inflammatory diseases, such as autoimmune diseases, neurodegenerative disorders, graft-versus-host disease, cancer, and atherosclerosis (reviewed by Schonbeck and Libby, (2001b). CD40 expression was up-regulated following *C. pneumoniae* infection, although there was variation between donors when cells were CM-treated (Figures 5.8 and 5.19). Up-regulation of CD40 may contribute to *C. pneumoniae*-mediated immunopathology. Higher expression of CD40 can contribute to Th1 type responses, and it can also up-regulate the expression of CD54 (ICAM-1) which can in turn enhance adhesion of cells and promote atheroma development (Kiener et al., 1995). Both treatments had a similar effect on CD54 expression, which was up-regulated in some donors and down-regulated in others (Figures 5.10 and 5.19). The up-regulation of CD54 may partly be a result of CD40 ligation; however this conclusion cannot be made from this study. Future studies could include functional analysis similar to the experiments suggested for CD91. Increased expression of CD40 and CD54 by the infected macrophage could increase the cell's pro-inflammatory responses and its pro-atherosclerotic properties.

Overall, there is donor variability in the expression of the molecules analysed and in the way that *C. pneumoniae* affects their expression. This may be due to a number of reasons. Since the blood is obtained from anonymous donors, there is no way of

knowing their medical history including previous chlamydial infections. Even though the blood bank performs a health check to ensure the donor is healthy at the time of giving blood, this is limited to colds, allergies and chronic conditions. Once the blood is collected there is an initial pathogen screen, however this does not include testing for chlamydial infection. Another important factor is the donor's genetic profile, which may contribute to the differences in the levels of surface molecule expression, to the donor's susceptibility to *C. pneumoniae* infections, and what symptoms they are likely to suffer. One way of overcoming this issue is to analyse a much larger donor base in order to obtain a more unified picture of how *C. pneumoniae* modulates immune responses of MdMs.

The quality of the Buffy coats and the isolation procedure on particular days may also have influenced the results. There are processes in the blood collection that are outwith experimental control, such as the length of time cells are sitting *ex vivo* before they are dispatched for research use, and this may affect the quality of each individual sample. Lastly, the fact that directly conjugated primary antibodies were used, may have affected the sensitivity of the analysis. For instance, TLR4 and CD91 consistently showed low-level expression. Multiple step analyses may reveal different results. The same is true of CD54 analysis in epithelial cells.

In summary, this study has shown that *C. pneumoniae* modulates the expression of a number of surface molecules involved in different aspects of building a specific, protective immune response. However *C. pneumoniae*-dependent modulation of surface molecules was only seen in MdMs, while infection did not have an effect on epithelial cells. In addition to the chosen molecules analysed (CD14, CD40, CD45, CD54, CD91, HLA ABC, HLA DR, TLR4) it is highly probable that *C. pneumoniae* affects the expression of many more molecules with different immune functions. These findings suggest that *C. pneumoniae* actively induces the immune response by modulation of both cytokine responses and the expression of surface molecules. However the final outcome of *C. pneumoniae* infection in each individual may also depend on a number of genetic and environmental factors.

CHAPTER 6

CONTROL OF CHLAMYDIAL GROWTH BY INFLAMMATORY CYTOKINES AND EXPRESSION OF SUPPRESSORS OF CYTOKINE SIGNALLING

6.1 *Introduction*

T helper 1 (Th1) cell-mediated immunity is important for host protection from *C. pneumoniae* infections, with T cell-derived IFN-γ playing a key role (Rottenberg et al., 1999, Penttila et al., 1998, Penttila et al., 1999). IFN-γ induces expression of indoleamine-2,3-dioxygenase (IDO), an enzyme that degrades intracellular pools of tryptophan (trp), an essential nutrient for chlamydial growth. Since the *C. pneumoniae* genome does not contain the *trp* operon (Kalman et al., 1999), degradation of tryptophan arrests chlamydial growth (Mehta et al., 1998, MacKenzie et al., 1999). Other cytokines such as TNF-α can synergise with IFN-γ to induce IDO expression (Summersgill et al., 1995).

The anti-chlamydial effects of IFN-γ are highly dose-dependent and do not necessarily result in eradication of the organism from host cells, but instead can result in persistence (Entrican et al., 2002). This finding points to a highly complex host-pathogen relationship where immune pressure can ultimately lead to long-term survival of the organism in a latent phase within host cells. Chlamydial persistence is often associated with severe immunopathology, and our current knowledge indicates that the responsiveness of host cells to inflammatory cytokines including IFN-γ is one of the key elements that define the outcome of infection (Rottenberg et al., 2002).

Inflammatory cytokines require tight host regulation to minimise the development of immunopathology. This can occur either by production of anti-inflammatory cytokines or by the induction of intracellular molecules that negatively control cytokine signalling in response to ligation of cell-surface receptors. Suppressors of cytokine signalling (SOCS) are a family of eight cytoplasmic molecules [SOCS1 to SOCS7 and

CIS (cytokine-inducible SH2-containing protein)] that are essential mediators for negatively regulating cytokine signalling. They share structural similarities and are characterised by a central SH2 domain and a unique motif, the SOCS box, in their C-terminal region (Hilton, 1999, Krebs and Hilton, 2001, Chen et al., 2000). SOCS have been detected in various tissues and are produced in response to different stimuli including cytokines, LPS and some bacterial infections (Stoiber et al., 2001, Stoiber et al., 1999, Lejeune et al., 2001, Losman et al., 1999, Ding et al., 2003, Dey et al., 2000, Dey et al., 1998, Hortner et al., 2002). SOCS regulate the magnitude and duration of responses triggered by various cytokines by inhibiting their signal transduction pathway in a classic negative feedback loop (Lang et al., 2003, Croker et al., 2003). SOCS interfere with Janus kinase/Signal Transducers and Activators of Transcription (Jak/Stat)-dependent signalling pathways. They bind directly to cytokine receptors or to the catalytic domain of Jak proteins and prevent the recruitment and phosphorylation of Stats. IFN-γ signalling is largely Jak/STAT dependent. Pathways activated by IFN-γ are described in Figure 6.1.

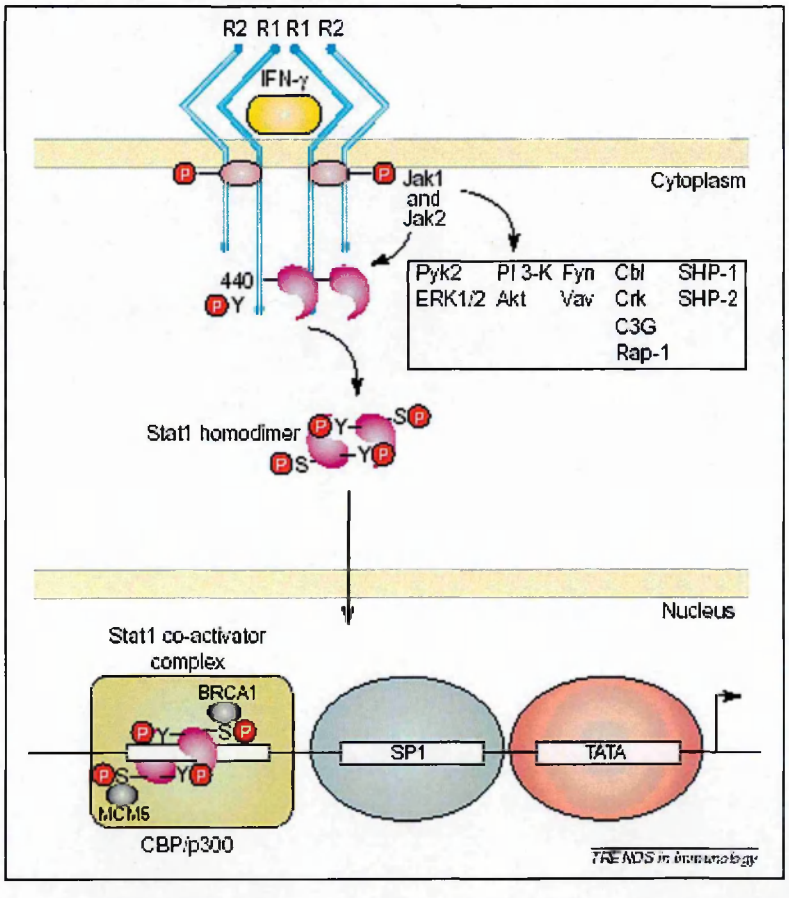


Figure 6.1 Signal-transduction pathways activated by IFN-γ. Ligand-induced oligomerization of the IFN-γ receptor subunits IFNGR1 and IFNGR2 leads to the phosphorylation and activation of Jak1, Jak2, IFNGR1 and Stat1. Stat1 homodimers translocate to the nucleus, bind to γ-activated sequence (GAS) elements and, in collaboration with other transcription factors (e.g.BRCA1 and MCM5), regulate gene expression. Several other signal-transduction pathways are activated also in parallel with the Jak–Stat1 pathway in response to IFN-γ (shown in the box). Pyk2 is a mitogen-activated protein tyrosine kinase; Vav, Cbl and Crk are adaptor proteins; C3G and Ras GTPase-activating protein 1 (Rap-1) are G-protein-linked signaling molecules; and Fyn is a Src-family tyrosine kinase. Abbreviations: BRCA1, breast cancer susceptibility gene 1; ERK, extracellular-signal-regulated kinase; Jak, Janus kinase; MCM5, mini-chromosome maintenance protein 5; PI 3-K, phosphatidylinositol 3-kinase; R1, IFNGR1; R2, IFNGR2; SHP, Src-homology-2-domain-containing protein tyrosine phosphatase; Stat1, signal transducer and activator of transcription 1. This Figure copied from Ramana et al., (2002) with permission from Elsevier Science.

SOCS1 and SOCS3 have both been associated with IFN-γ signalling (Song and Shuai, 1998, Stoiber et al., 1999). SOCS1 binds to Jak1 and Jak2, which are associated with IFN-γ R subunits 1 and 2 respectively. This then prevents STAT1 phosphorylation and subsequent activation of IFN-γ responsive genes. SOCS3 also inhibits IFN-signalling through inhibition of STAT1 activation, but the effects are weaker (Stoiber et al., 1999, Cacalano et al., 2001, Woldman et al., 2001, Kovarik et al., 1998). The mechanisms of SOCS activity have been extensively studied but there have been a limited number of reports on their expression and role in pathological conditions and infections (Dogusan et al., 2000, Federici et al., 2002, Hong et al., 2002, Hong et al., 2001, Seki et al., 2003, Raccurt et al., 2003).

Previous work in the laboratory has shown that certain lung epithelial ceils differ substantially in their ability to control chlamydial growth in response to IFN-γ treatment. Unresponsiveness of lung epithelial cells to IFN-γ could have serious implications for the outcome of chlamydial infection and the induction of protective immunity. In this chapter experiments designed to clarify the mechanisms of this phenomenon are presented. They were designed to:

- Investigate the ability of epithelial cells to control chlamydial growth in response to IFN-γ and investigate the induction of IDO, a control mechanism for *C. pneumoniae*
- Investigate the expression of SOCS1 and SOCS3 in lung epithelial cells in response to IFN-γ and chlamydial infection, with a view to better understand the mechanisms of chlamydial persistence as a result of immunological pressure.

6.2 ***Experimental approach***

Methods used in this chapter are described in detail in Chapter 2.

For analysis of mRNA expression in all experiments the cells were seeded in 24 well plates at $2x10^5$ cells/well in 1 ml of IMDM supplemented with 5% FCS. Cells were left overnight to adhere and were treated the following day. Stimuli added were always diluted in IMDM supplemented with 2% FCS. Samples were collected at different times after treatment and RNA extracted (section 2.3.1). Gene expression was analysed either by standard RT-PCR (section 2.2.3) or Real Time RT-PCR (section 2.2.4).

Surface expression of IFN-γ receptor was analysed by flow cytometry (section 2.3.2).

For western blotting analysis of SOCS3 protein, cells were seeded in $75cm^2$ flasks at $0.8x10^6$/ml in 10 ml of medium. The preparation of the lysates and western blot analysis is described in section 2.5.

All *in situ* immunohistochemistry and immunofluorescence experiments were done on 8 well chamber slides. Cells were seeded at $1x10^5$ cells/ml at 600µl per well. Cells were left to adhere overnight and were treated the following day. Methods are described in detail in the following sections: DAB immunohistochemistry, section 2.6.1; Single immunofluorescence, section 2.6.2.1; Double immunofluorescence, section 2.6.2.2.

6.3 *Results*

6.3.1 Control of chlamydial growth in lung epithelial cells in response to IFN-γ

The ability of IFN-γ to induce anti-chlamydial responses in lung epithelial cells was investigated. HEp-2 and A549 cells were treated with 500 U/ml of IFN-γ at various times in relation to infection with MOI=1 of *C. pneumoniae*: 24 hours prior to infection, at the time of infection, and 24 hours after infection, in order to investigate the ability of the cells to control the growth in response to IFN-γ. HEp-2 cells controlled the growth of *C. pneumoniae* when they were treated with IFN-γ prior to infection and not in any other case (Figure 6.2 b). The effect of IFN-γ treatment on chlamydial growth will be discussed further in Section 6.3.7.3. A549 cells did not control chlamydial growth in response to IFN-γ at all (Figure 6.2 e). The inclusions in A549 cells treated with IFN-γ were similar in size and appearance to untreated cells at all time points (compare Figure 6.2 d and e).

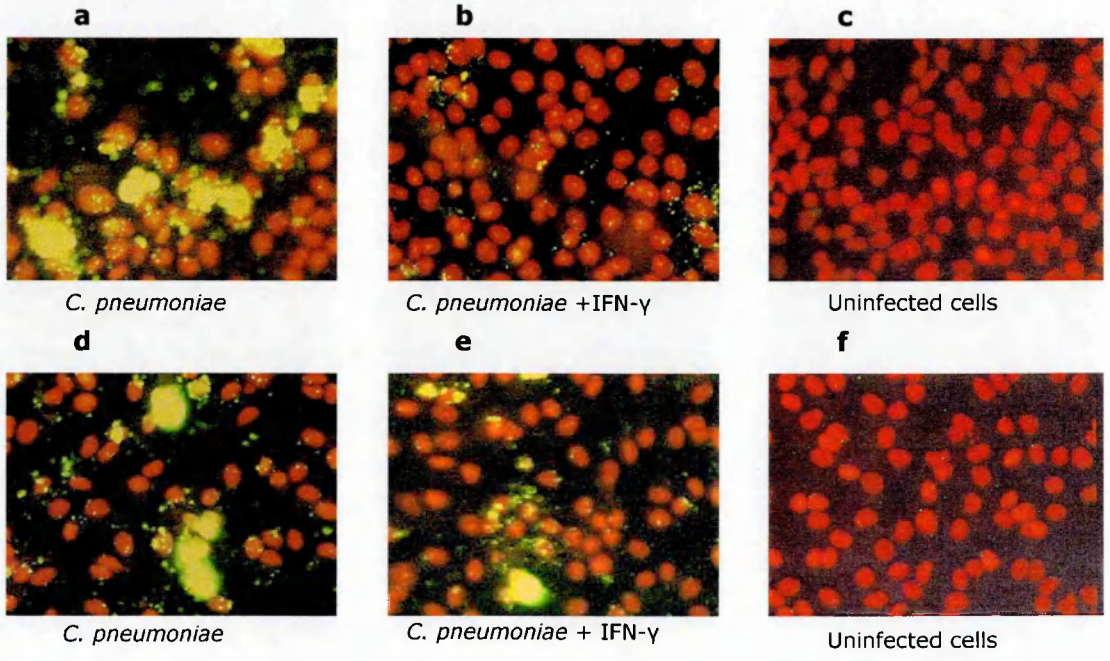


Figure 6.2 Control of chlamydial growth by epithelial cells in response to inflammatory cytokines. Cells were treated with 500 U/ml of IFN-γ for 24 hours prior to infection with *C. pneumoniae* (MOI=1). Cells are stained for chlamydial LPS (**green**) and the nuclei are counterstained in (**red**). Upper panel HEp-2 cells, lower panel A549 cells. Original magnification 200x.

The inability of A549 cells to control chlamydial growth in response to IFN-γ was a surprising finding. This may be due to a number of reasons, one of them being the lack of IFN-γ receptor on the cell surface, or an inhibition of IFN-γ-dependent signalling pathways. In order to address this phenomenon I decided to start by analysing the expression of IFN-γ receptor on both cell types.

6.3.2 Expression of IFN-γ receptor on A549 and HEp-2 cells

Both A549 and HEp-2 cells were found to express comparable levels of IFN-γ receptor on their surface, and *C. pneumoniae* infection does not alter the expression (Table 6.1 and Figure 6.3). The expression was analysed by flow cytometry and MFIs are presented in Table 6.1. From MFI data it seems that the expression is down-regulated; however the histograms show that the distribution of cells is very spread out rather than a normal distribution (Figure 6.3) and any apparent shift in MFI may have a limited value in explaining the change of IFN-γ receptor expression.

Table 6.1 Mean fluorescence intensities of IFN-γ receptor expression on A549 and HEp-2 lung epithelial cells following *C. pneumoniae* infection.

Molecule	Cells	IFN-γ receptor			
		6hr	24hr	48hr	72hr
A549	Uninfected	170.83	504.81	229.27	99.70
	Infected	127.70	416.66	78.21	99.76
HEp-2	Uninfected	151.89	257.94	73.38	49.09
	Infected	105.43	187.62	59.43	40.28

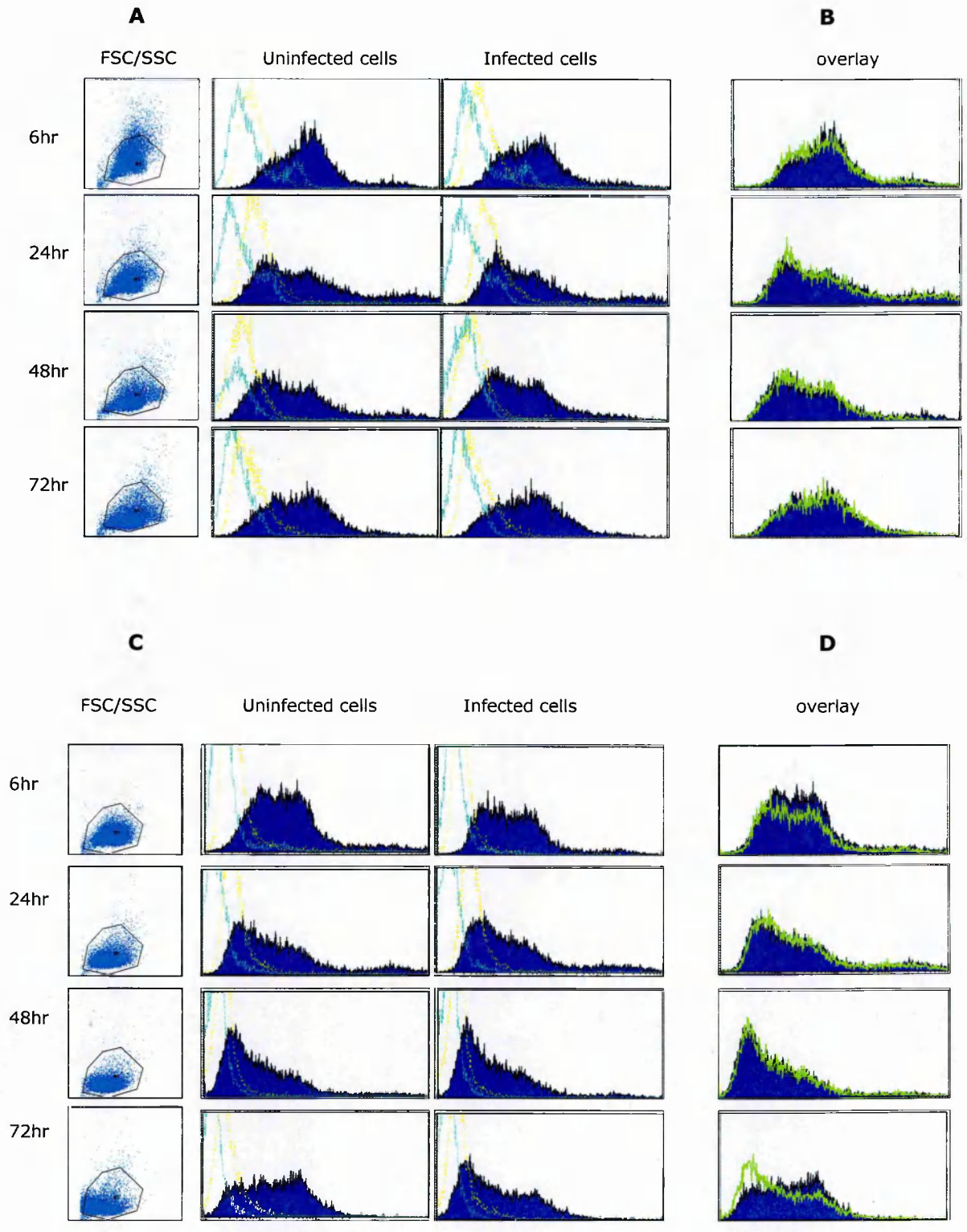


Figure 6.3 IFN-γ R expression on A549 and HEp-2 cells following *C. pneumoniae* infection. Cells were infected with *C. pneumoniae* (MOI=1) and samples were collected 6, 24, 48 and 72 hours after infection **(A)** A549 cells **(B)** A549 cells overlay of infected and uninfected cells **(C)** HEp-2 cells **(D)** HEp-2 cells overlay of infected and uninfected cells. Key: **purple field**-IFN-R; **yellow line** – 2nd step control; **blue line** - 3rd step control. In the overlay: **purple field** – uninfected cells; **green line** - infected cells.

Given that both A549 and HEp-2 cell lines express IFN-γ receptor, their responsiveness to IFN-γ treatment was tested. Expression of IDO following IFN-γ treatment is a central mechanism of control of chlamydial growth and it has been demonstrated previously that lack of IDO expression in response to IFN-γ correlates with the lack of control of chlamydial growth (Entrican et al., 2002). IDO expression was therefore chosen as a marker of IFN-γ responsiveness.

6.3.3 Expression of IDO mRNA in epithelial cells in response to IFN-γ treatment

Expression of IDO mRNA was analysed by RT-PCR in both A549 and HEp-2 cells. Neither cell line was found to express IDO mRNA in the resting state, whereas treatment of cells with 500 U/ml of IFN-γ for 24 hours induces IDO mRNA expression in both HEp-2 and A549 cells (Figure 6.4).

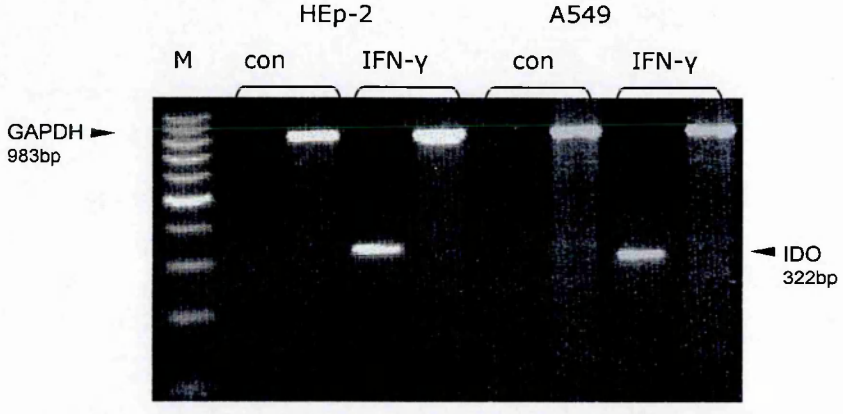


Figure 6.4 Expression of IDO mRNA in response to IFN-γ treatment in lung epithelial cells. Cells treated with 500 U/ml of IFN-γ for 24 hours. Data representative of 5 experiments. Key: M=marker; con=untreated cells; IFN-γ= IFN-γ treatment.

The fact that A549 cells are responsive to IFN-γ insofar as they express IDO mRNA, yet cannot control chlamydial growth was an unexpected and exciting finding. Taking all of the findings into consideration the implication is that IFN-γ signalling is inhibited in A549 cells, I decided to investigate the expression of SOCS1 and SOCS3 in A549 and HEp-2 cells.

6.3.4 Analysis of SOCS1 and SOCS3 expression in resting cells by PCR

SOCS1 and SOCS3 have both been shown to interfere with IFN-γ signalling and therefore their expression was examined in HEp-2 and A549 cells by RT-PCR. The primers were designed using the PrimerSelect program within the DNAStar Version 5.06 sequence analysis package (DNAStar; GATC Biotech AG, Konstanz, Germany). Initial attempts to detect SOCS1 and SOCS3 were inconclusive with a lot of non-specific bands. SOCS3 but not SOCS1 was detected in A549 cells, while HEp-2 did not seem to express either (Figure 6.5.A). In order to obtain a more sensitive reaction and a cleaner PCR product, a nested PCR was optimised for SOCS3 analysis. The new reaction was a combination of two 25 cycle PCR reactions using a set of 'outer' and 'inner' primer pairs, respectively. The new reaction proved to be more sensitive and now SOCS3 mRNA was also detected in HEp-2 resting cells (Figure 6.5.B).

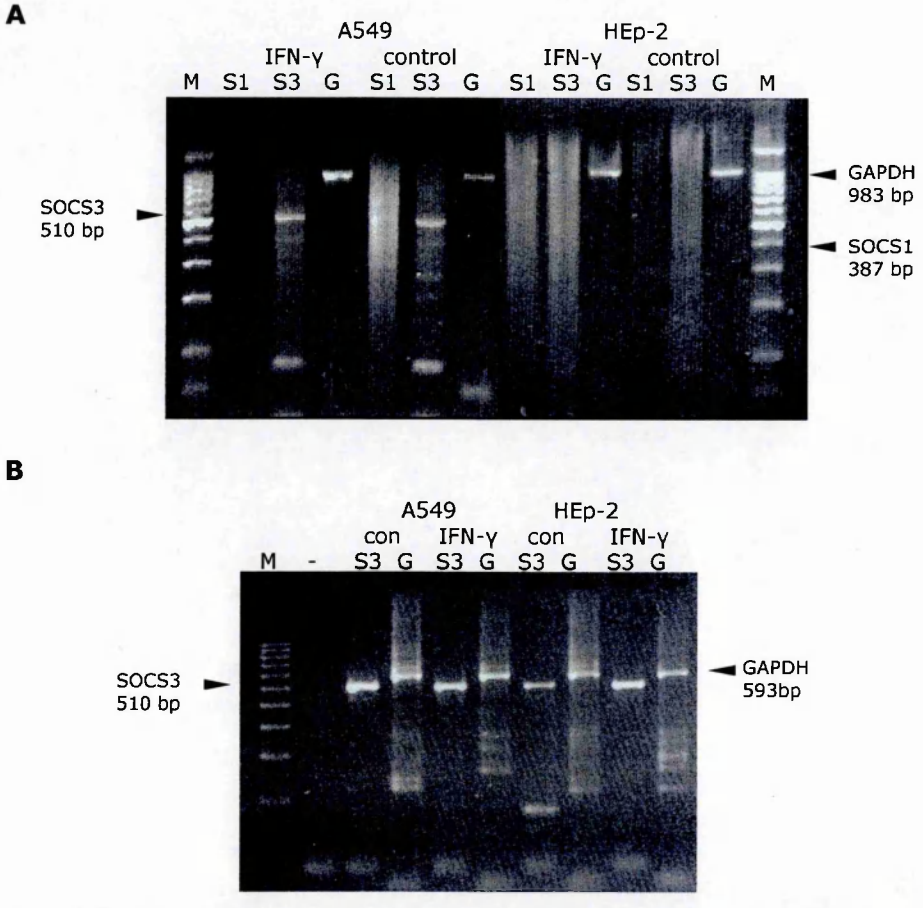


Figure 6.5 Optimisation of a nested PCR for detection of SOCS. **(A)** initial attempts with a single step PCR for SOCS1 and SOCS3; **(B)** optimised nested PCR for SOCS3. Key: M-marker, S1-SOCS1, S3-SOCS3, G-GAPDH; IFN-γ – cells treated with 500 U/ml for 24 hours; con – untreated cells.

To confirm the results of the nested PCR and to further analyse SOCS1 mRNA expression, a new set of primers were obtained for a single step PCR for both SOCS1 and SOCS3 (kind gift from Prof Jim Johnston, Queens University, Belfast). This was a single step PCR with 40 cycles. With this reaction previous results of SOCS3 expression in resting cells were confirmed (Figure 6.6 A). When cells were infected with *C. pneumoniae* there is a suggestion of a change in expression of SOCS3 (Figure 6.6 A); however given that the target gene is expressed in resting cells, the differences are difficult to quantify using a standard RT-PCR.

Very faint bands of SOCS1 mRNA are visible in resting HEp-2 cells and cells infected with *C. pneumoniae* (Figure 6.6 B HEP-2 columns 4, 1 and 2 respectively).
In A549 cells there is no SOCS1 in resting cells but there seems to be up-regulation of mRNA expression in response to *C. pneumoniae* (Figure 6.6 B A549 column 4, 1 and 2 respectively).

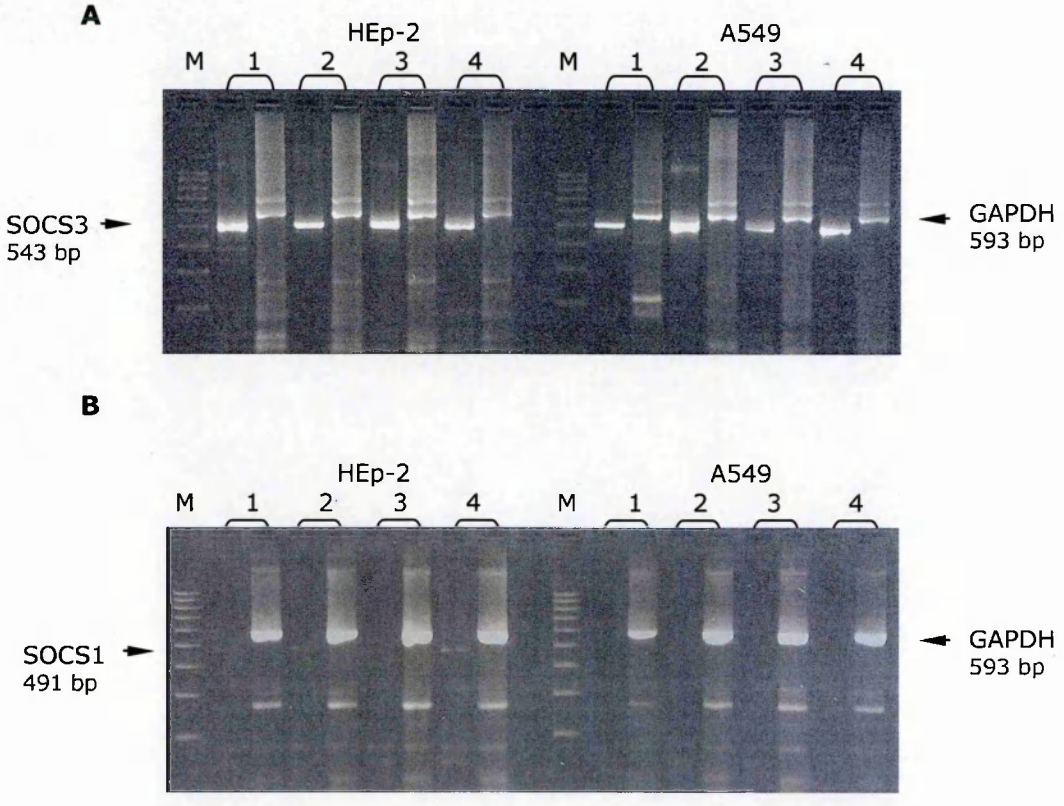


Figure 6.6 SOCS1 and SOCS3 mRNA expression in epithelial cells in the first 24 hours after the infection. **(A)** SOCS3 **(B)** SOCS1. Columns: 1- *C. pneumoniae* MOI=1; 2- *C. pneumoniae* MOI=10; 3- IFN-γ 500 U/ml and *C. pneumoniae* MOI=1; 4-uninfected cells.

These results indicated that SOCS3 is expressed constitutively in both HEp-2 and A549 cells irrespective of their ability to control chlamydial growth. Since SOCS1 and SOCS3 can negatively regulate IFN-γ signals it was interesting to investigate whether *C. pneumoniae* infection can change the level of SOCS3 expression and whether this is influenced by IFN-γ treatment of cells prior to infection.

6.3.5 *C. pneumoniae* infection up-regulates SOCS3 mRNA expression in lung epitheliai cells

To analyse the change in SOCS3 mRNA expression quantitatively, Real time RT-PCR was used. Cells were infected with multiplicity of infection of 1 or 10 over 72 hours. There was a time and dose-dependent up-regulation of SOCS3 mRNA expression in both HEp-2 and A549 cells following *C. pneumoniae* infection (Figure 6.7). SOCS3 mRNA is up-regulated in the first 6 hours with two peaks, one between 1 and 2 hours and another at 6 hours. In A549 cells mRNA expression returns to background levels after 6 hours (Figure 6.7 A) while in HEp-2 cells SOCS3 mRNA expression is up-regulated over 72 hours (Figure 6.7 C). In order to identify the component(s) responsible for the effect of *C. pneumoniae* on SOCS3, as a first step the effects of LPS were analysed. Cells were treated with 1 μg/ml of *S. minnesota* LPS which is similar to chlamydial LPS both in structure and endotoxic activity. *S. minnesota* LPS does not induce SOCS3 mRNA expression (Figure 6.7 A and D; experiment not repeated).

Results described here show that *C. pneumoniae* does change the level of SOCS3 expression and it does so independently of LPS. Promoting SOCS3 expression in lung epithelial cells could render them less responsive to IFN-γ and this could in turn contribute to long term infection. Since there are speculations that IFN-γ can also contribute to persistence the next step was to investigate whether IFN-γ alters SOCS3 expression patterns of infected cells and whether it can induce SOCS3 expression on its own.

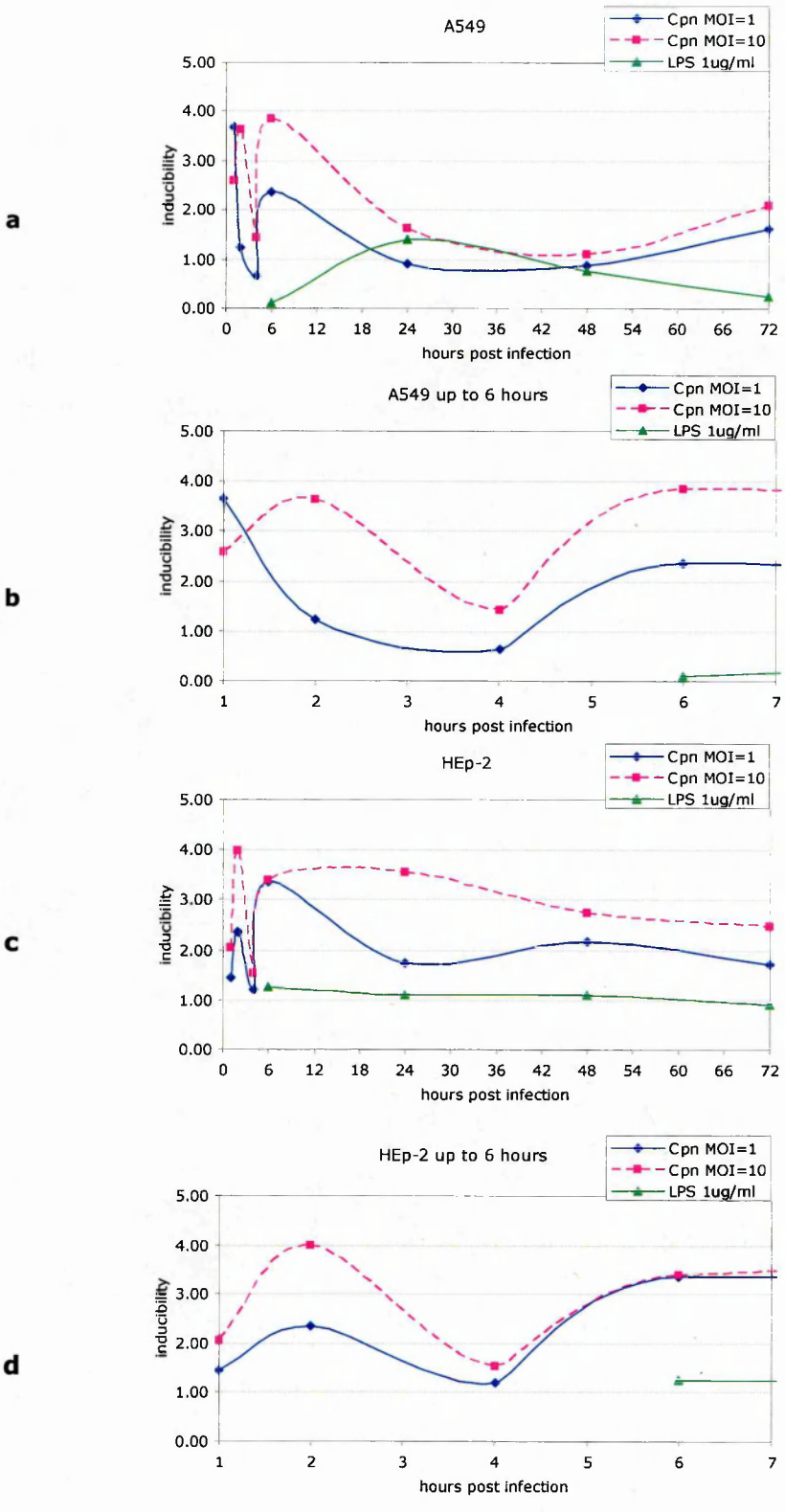


Figure 6.7 Expression of SOCS3 mRNA in response to *C. pneumoniae* infection and *S. minnesota* LPS treatment**. (A)** A549 cells infection over 72 hours **(B)** A549 cells, infection over 6 hours **(C)** HEp-2 cells, infection over 72 hours **(D)** HEp-2 cells infection over 6 hours. Cells were infected with *C. pneumoniae* (MOI=1 (♦) or 10 (■) or treated with 1µg/ml of LPS (▲) and samples were analysed up to 72 hours. Analysis was done in duplicates, data expressed as mean inducibility of SOCS3 mRNA over untreated cells at each time point. Inducibility – fold increase or SOCS3 expression of treated cells over untreated cells. Data representative of three separate experiments (only one experiment done for LPS).

6.3.6 The effect of IFN-γ on SOCS3 mRNA expression

To further explore the responsiveness of epithelial cells to IFN-γ they were treated with 500 U/ml of IFN-γ alone or in combination with MOI=1 of *C. pneumoniae* over 72 hours, and expression of SOCS3 mRNA was analysed (Figure 6.8). IFN-γ unresponsive A549 cells showed up-regulation of 1.9 fold of SOCS3 expression when treated with IFN-γ alone, with a peak at 24 hours post treatment. This change is not considered biologically significant. To be considered biologically significant, a change of less than 0.5 (down-regulation), and greater than 2 fold (up-regulation) must be recorded in two separate experiments (Chtanova et al., 2001).

When the cells were pre-treated with IFN-γ for 24 hours and then infected with *C. pneumoniae* at MOI=1, there was a peak of SOCS3 mRNA expression at 2 hours after infection and then again at 6 hours which is similar to the pattern seen with *C. pneumoniae* infection alone. There was induction again at 72 hours (2.3 fold).

HEp-2 cells showed steady levels of SOCS3 mRNA over 72 hours in response to IFN-γ treatment (~2.5 fold). When treated with both IFN-γ and *C. pneumoniae* there was a synergy of the two treatments in inducing SOCS3 mRNA expression, and the pattern of expression resembled the pattern of *Chlamydia* infection only (see Figure 6.7 B).

Analysis of SOCS3 mRNA expression has shown that it is expressed constitutively and *C. pneumoniae* infection up-regulates its expression. IFN-γ causes further up-regulation of SOCS3 mRNA expression in HEp-2 cells while in A549 cells the effects are weaker. Following these findings it was important to analyse the expression of SOCS3 protein to find out whether these treatments have an effect on the protein expression and whether SOCS3 affects STAT1 phosphorylation inhibiting IFN-γ-mediated functions in this way.

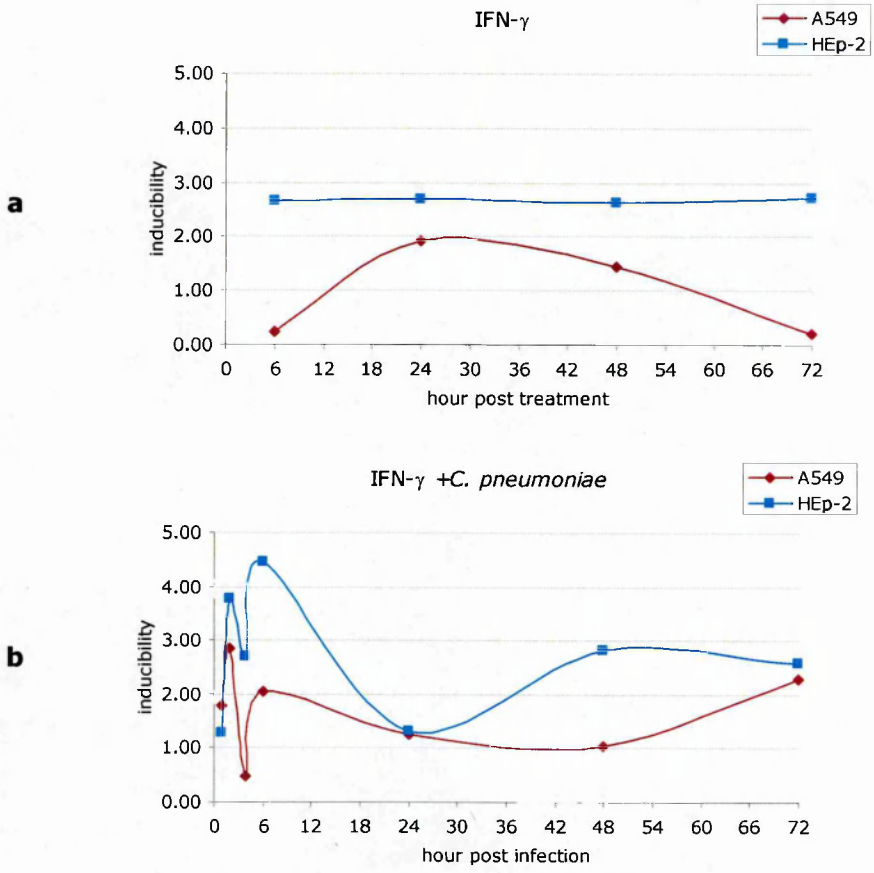


Figure 6.8 Expression of SOCS3 mRNA in response to IFN-γ and *C. pneumoniae*. **(A)** IFN-γ treatment. Cells were treated with 500 U/ml of IFN-γ over 72 hours. **(B)** IFN-γ treatment and *C. pneumoniae* infection. Cells were pre-treated with 500 U/ml of IFN-γ for 24 hours and then infected with *C. pneumoniae* MOI=1. Samples were collected and analysed up to 72 hours after the infection. Analysis done in duplicate, data expressed as mean inducibility over untreated cells at each time point. Inducibility – fold increase or SOCS3 expression of treated cells over untreated cells Data representative of three separate experiments.

6.3.7 Analysis of SOCS protein expression in lung epithelial cells

In the view of the findings described above there were several key questions regarding SOCS protein expression:

Are SOCS proteins expressed in resting cells?

Is SOCS protein expression changed by *C. pneumoniae* infection?

Does IFN-γ pre-treatment of infected cells influence SOCS protein expression?

Where in the cells do the SOCS proteins localise, and does this change with treatment?

To address these questions three different techniques were used to detect SOCS proteins: Western Blotting, DAB (diaminobenzidine) immunohistochemistry analysed by light microscopy, and immunofluorescence analysed by confocal microscopy.

6.3.7.1 Analysis of SOCS protein expression by Western Blotting

At the time of planning these experiments there were a limited number of reagents available for detection of SOCS at the protein level. Initial attempts to get any specific staining for either SOCS1 or SOCS3 failed. A number of different conditions were changed without success (different lysis buffer; increased concentration of protein; longer blotting time; different blocking reagent; increased concentrations of antibodies with longer incubation times; longer exposure of the film to ECL). Considering the lack of SOCS1 mRNA expression in both cell lines, SOCS1 protein analysis by western blot was abandoned completely.

In order to solve the problem of SOCS3 protein expression, I initiated a collaboration with Professor Jim Johnston at the Department of Immunology and Microbiology, Queens University Belfast. The focus of his research is SOCS proteins and their functions in immunology (Cacalano et al., 2001, Hortner et al., 2002, Seki et al., 2003, Haan et al., 2003). I visited Professor Johnston's laboratory for a week in March 2003 where I analysed SOCS3 expression using an antibody optimised for western blotting. A549 and HEp-2 cells were infected with *C. pneumoniae* at MOI=1 or pre-treated with 500 U/ml of IFN-γ for 24 hours and then infected. Whole cell lysates were prepared with equal amounts of lysis buffer and equal amounts of protein were used for analysis. Some of the protein was immunoprecipitated using agarose beads and anti-SOCS3 rabbit polyclonal antibody (in-house, QUB, Belfast). The results are presented in Figure 6.9.

Analysis of whole cell lysates shows that A549 cells express SOCS3 protein constitutively while HEp-2 cells do not. There is an up-regulation of SOCS3 protein in both cell lines after infection with *C. pneumoniae*. In HEp-2 cells the peak is between 24 and 48 hours after the infection. IFN-γ pre-treatment of the cells seems to down-

regulate SOCS3 expression compared to *C. pneumoniae* only in the first 48 hours. This differs from the Real time RT-PCR results. Unfortunately the HEp-2 sample for IFN-γ treatment and infection was destroyed in transit and was not analysed (Figure 6.9 A).

In A549 cells there is up-regulation of SOCS3 protein expression for the whole 72 hour period with no difference with IFN-γ treatment (Figure 6.9 B).

When SOCS3 expression is analysed by immunoprecipitation, there does not seem to be a change of expression in either cell line. However, the immunoprecipitation protocol did not work well; there was a lot of background when compared to the positive control. The protocol needed optimisation, and given that the whole cell lysates showed promising results the immunoprecipitation approach was abandoned.

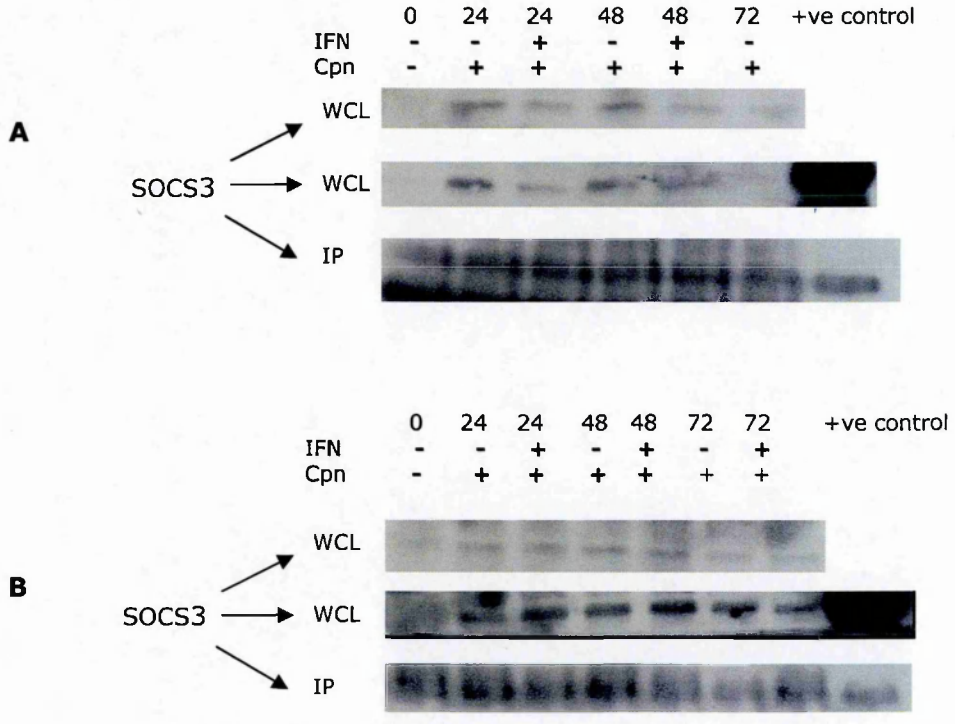


Figure 6.9 Western blot analysis of SOCS3 protein expression done in Belfast (Mar 2003). **(A) HEp-2 cells (B) A549 cells**. Blots were repeated twice for whole cell lysates with different amounts of lysate. Times refer to hours after infection. WCL-whole cell lysates; IP-immunoprecipitate; IFN-γ – cells treated with 500 U/ml IFN-γ; C.pn – cells infected with MOI=1 of *C. pneumoniae*; +ve control-recombinant SOCS3.

Further analysis was conducted on SOCS3 protein expression in response to IFN-γ and *S. minnesota* LPS treatment, and the previous experiment was repeated to confirm the results obtained in Prof. Johnston's laboratory. The whole cell lysates were prepared in the same way as before, and the protein concentration was measured. A

maximum of 120 µg of protein was put on the gel. As seen in Figure 6.10 the results were inconsistent. The amount of protein detected in different blots differed even though the same amounts of protein were loaded onto the gel. However the β-actin bands are similar in thickness. These results indicate that *C. pneumoniae* treatment does not induce a change in expression which is in conflict with both Real time RT-PCR and the western blot results described above. In the case of IFN-γ and LPS treatment the levels of background production were different from those found with *C. pneumoniae* alone and there was no consistency between the bands so it was not possible to draw conclusions.

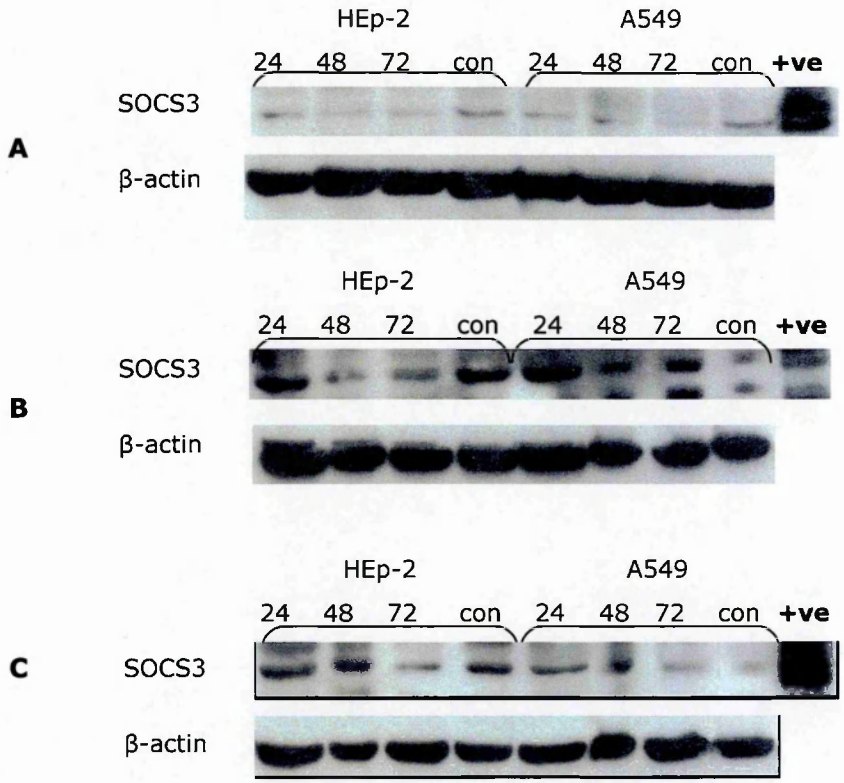


Figure 6.10 Western blot analysis of SOCS3 expression in response to different treatments. **(A)** *C. pneumoniae* infection MOI=1 **(B)** IFN-γ treatment 500 U/ml **(C)** LPS treatment 1 µg/ml. Numbers are hours of treatment.

Any one of a number of reasons could explain the discrepancies in the results obtained in different labs: different gel system; different buffer; different transfer system - a semi-dry blotter was used in both cases, but they were from different companies. All of this may have affected the binding efficiency of the SOCS antibody. Taking into account all the results presented here and all the inconsistencies encountered it would seem that western blotting was not an ideal technique for

analysis of differences in SOCS3 protein expression. It did, however, establish that both A549 and HEp-2 cells express SOCS3 protein.

Analysis of SOCS1 protein expression gave negative results and, given the PCR results, it is likely that SOCS1 protein is not expressed in either A549 or HEp-2 cells.

Western blotting is limited to revealing the presence of a protein in the cell lysate and it is easily affected by the method and quality of the cell lysates. It does not give any information about the localisation of the target protein in the cell. Given that the results indicated the presence of the protein in both cell lines, the next logical step was to investigate where in the cell SOCS3 is localised and whether the localisation changes in response to different treatments. These questions were addressed with *in situ* analysis by immunohistochemistry.

6.3.7.2 Analysis of SOCS protein expression by immunohistochemistry

The localisation of SOCS proteins in the cell was analysed by immunohistochemistry. Initially a biotin/avidin/DAB staining was used and analysed by light microscopy (section 2.6.1). Cells were treated with (I) *C. pneumoniae* MOI=1 and MOI=0.1 and stained at 72 hours after infection; (II) 500 U/ml of IFN-γ at different times in relation to the infection with *C. pneumoniae* MOI=1; (III) 500 U/ml of IFN-γ alone up to 72 hours. Staining for SOCS1 protein was completely negative in both cell types.

C. pneumoniae induces SOCS3 protein expression in both A549 and HEp-2 cells, in a dose-dependent manner (Figure 6.11 and 6.12 B and C). 72 hours after the infection, when the inclusions are fully developed, SOCS3 protein is concentrated at the site of the inclusion. When HEp-2 cells are pre-treated with IFN-γ, SOCS3 protein expression is down-regulated (Figure 6.11 D). When they are treated with IFN-γ at the time of the infection there is some suppression and if treated with IFN-γ 24 hours after the infection the pattern of expression is comparable to untreated infected cells (Figure 6.11 E and F respectively). The expression of SOCS3 protein in infected A549 cells does not change in response to IFN-γ treatment and the pattern of expression is similar to that of infected cells (Figure 6.12 D to F).

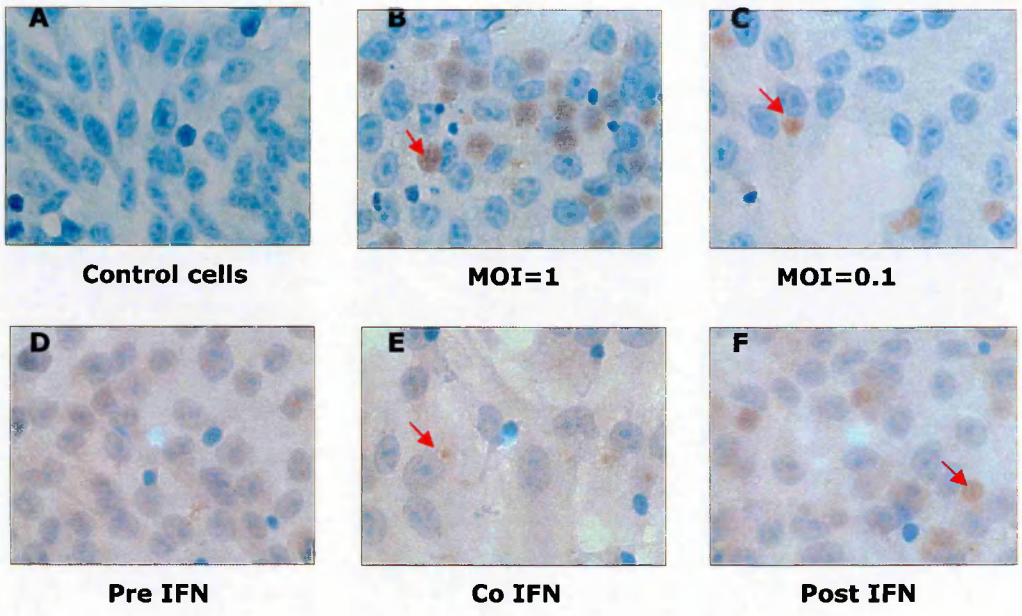


Figure 6.11. SOCS3 protein expression in infected HEp-2 cells analysed by light microscopy. Cells were infected with *C. pneumoniae* and treated with 500 U/ml of IFN-γ at different times in relation to infection. SOCS3 is stained brown with DAB. Examples of staining are indicated with red arrows. Nuclei were counterstained blue with hematoxylin. Key: Pre IFN - treatment 24 hr prior to infection with MOI=1; Co IFN-treatment at the time of the infection with MOI=1; Post IFN –treatment 24 hr after the infection with MOI=1. Original magnification 200x.

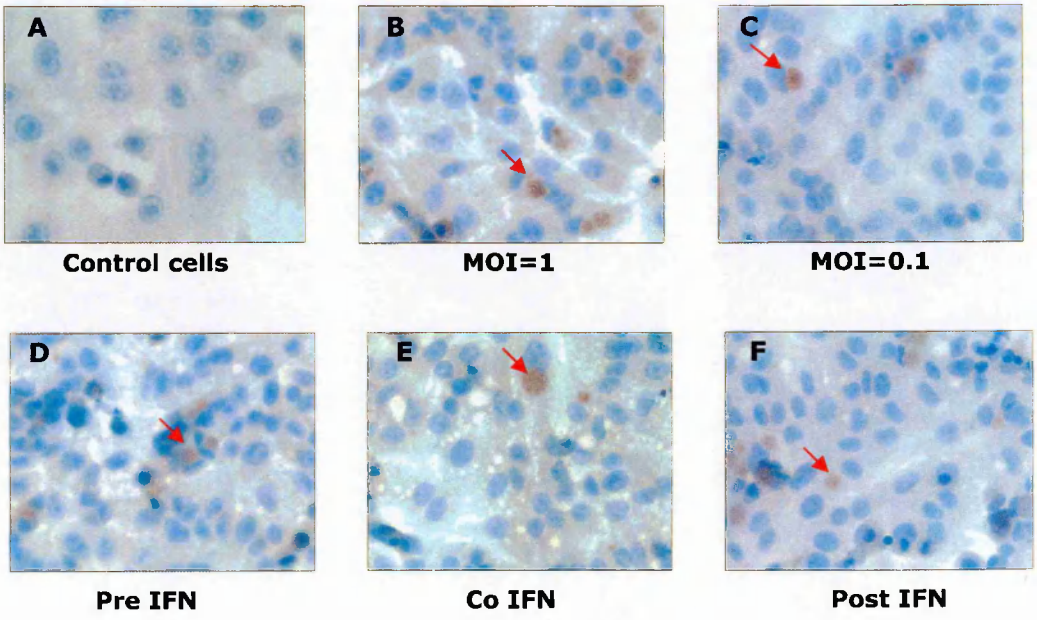


Figure 6.12. SOCS3 protein expression in infected A549 cells analysed by light microscopy. Cells were infected with *C. pneumoniae* and treated with 500 U/ml of IFN-γ at different times in relation to infection. SOCS3 is stained brown with DAB. Examples of staining are indicated with red arrows. Nuclei were counterstained blue with hematoxylin. Key: Pre IFN - treatment 24 hr prior to with MOI=1; Co IFN-treatment at the time of the infection with MOI=1; Post IFN – treatment 24 hr after the infection with MOI=1. Original magnification 200x.

IFN-γ treatment of A549 cells up-regulates SOCS3 two hours after treatment and the protein is localised in the cytoplasm of the cells (Figure 6.13 B). This goes back to background levels at 4 hours and there does not seem to be a further change of expression over the 72 hour period (Figure 6.13 C to G). The darker patches in the 48 and 72 hour panels could be an artefact of cell density rather than true staining (Figure 6.13 F and G). IFN-γ can exhibit an anti-proliferative effect in certain cells; however as can be seen in these panels there is no growth inhibition in A549 cells over the 72 hours of treatment. This may be another indicator of the partial responsiveness of A549 cells to IFN-γ treatment. IFN-γ did not induce SOCS3 expression in HEp-2 cells, but there is growth arrest as seen in Figure 6.14. HEp-2 cells were only analysed at 24 hours and later. Considering the results obtained from A549 cells this should be included in further studies.

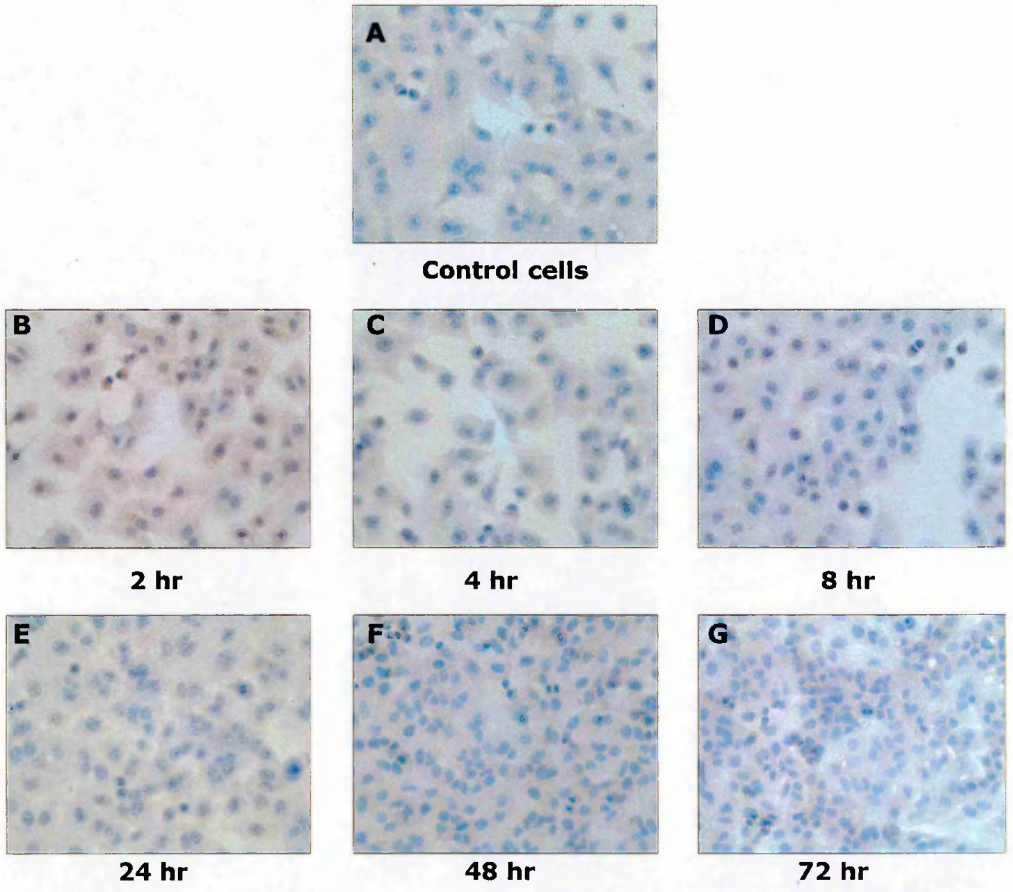


Figure 6.13 A549 cells stained for SOCS3 protein in response to IFN-γ treatment over 72 hours. Cells were grown on 8 well chamber slides, and staining performed as described (section 2.6.1). SOCS3 is stained brown with DAB. Nuclei are counterstained blue with haematoxylin Data representative of four experiments. Original magnification 200x.

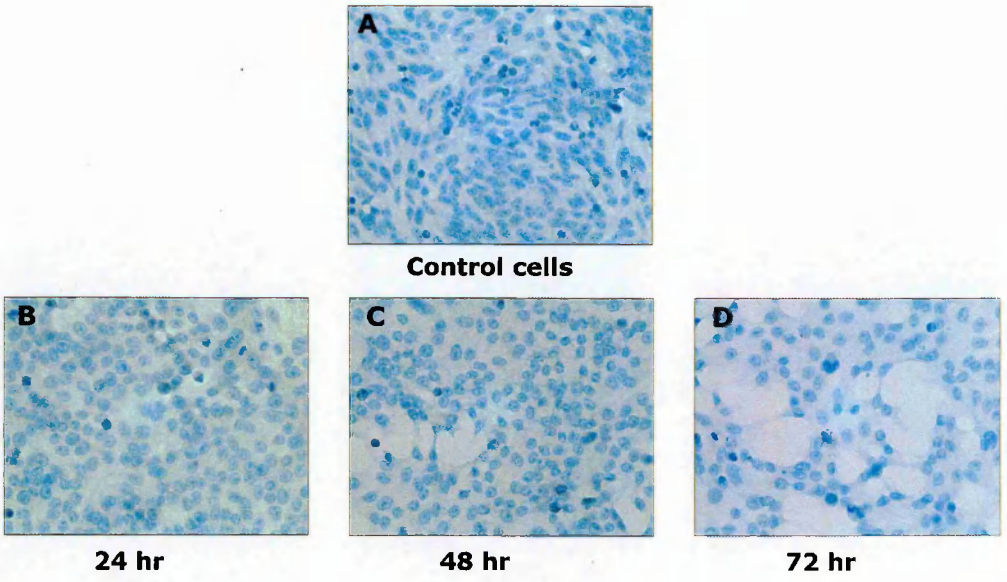


Figure 6.14 SOCS3 expression in HEp-2 cells over 72 hours. Cells were treated with 500 U/ml of IFN-γ and stained at different time points. SOCS3 is stained brown with DAB. Nuclei are counterstained blue with haematoxylin. Data representative of at least 3 experiments. Original magnification 200x.

Constitutive expression of SOCS3 protein was not detected in either cell line by DAB immunohistochemistry. It may be that the levels of SOCS3 protein are beyond the sensitivity of this technique. What is very interesting is the induction of SOCS3 protein expression in infected cells 72 hours after the infection. The pattern of infection resembles chlamydial inclusions; however this could not be confirmed by this method. In the light of this it was decided to continue SOCS3 protein analysis by immunofluorescence and confocal microscopy that should be more sensitive techniques.

6.3.7.3 Analysis of SOCS protein expression by confocal microscopy

To confirm the results obtained by DAB staining and to analyse the localisation of SOCS3 protein in relation to chlamydial inclusions SOCS3 expression was further analysed by confocal microscopy (method described in section 2.6.2.2). Cells were infected for 72 hours and were treated with IFN-γ at different times in relation to infection as described in the previous section. Cells were also infected for 6 hours to investigate the early expression of SOCS3 protein in response to infection. Samples were stained for chlamydial LPS to locate the inclusion and for SOCS3. The results are

presented as confocal images along with a corresponding diagram. The diagrams represent the pixel intensity of each channel of the measured section on the image; this graphic form gives a clearer representation for analysis of protein localisation. The intensity value is an arbitrary number with a range 0-256; values under 20 are considered as background.

No expression of SOCS3 protein could be demonstrated in resting HEp-2 cells (Figure 6.15 A). Infection over 6 hours induces some SOCS3 protein expression and the protein localises in the cytoplasm and this does not change at 72 hours (Figure 6.15 B and C) and this can be better seen in the accompanying graph.

When HEp-2 cells are treated with IFN-γ the intensity of LPS staining and the size of inclusions changes, depending on the time of treatment in relation to the infection (Figure 6.16). Levels of SOCS3 staining change accordingly. The strongest intensity is visible in image 6.16 C where IFN-γ was added 24 hours after the infection.

A549 cells have some background SOCS3 expression in resting cells (Figure 6.17 A). This is enhanced after 6 hours of infection (Figure 6.17 B). SOCS3 protein is again localised in the cytoplasm. 72 hours after the infection SOCS3 is still localised in the cytoplasm at similar levels (Figure 6.17 C). The levels of SOCS3 staining are higher in A549 cells than in HEp-2 cells for all the conditions.

IFN-γ treatment of A549 cells does not affect the staining patterns or levels of SOCS3 irrespective of when the cells were treated in relation to infection (Figure 6.18). Confocal analysis has also confirmed the initial finding that the IFN-γ does not control chlamydial growth in A549 cells and that the appearance and size of inclusions does not change in response to IFN-γ (discussed in Section 6.1).

Figure 6.15

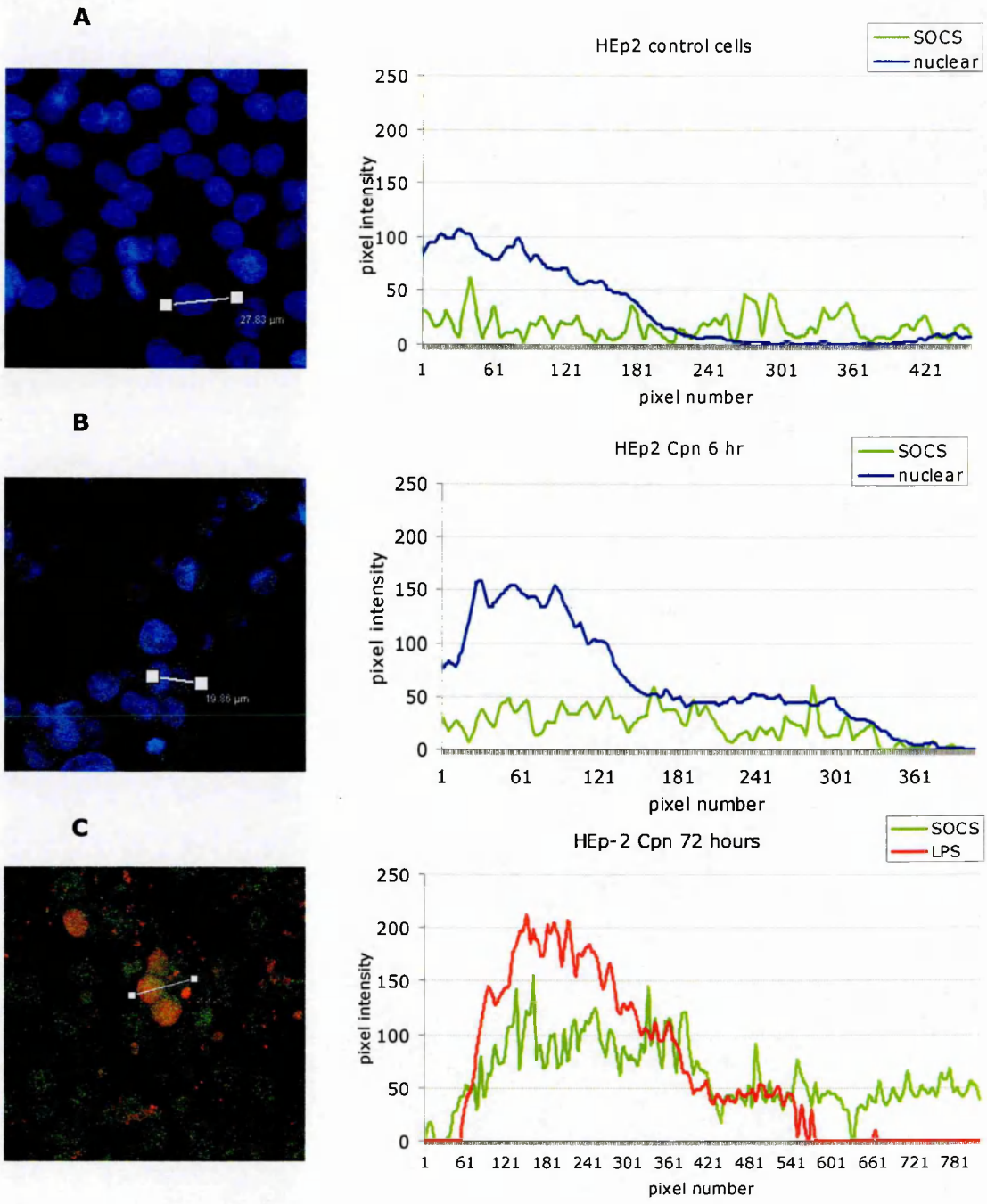


Figure 6.15 Expression of SOCS3 protein in HEp-2 cells analysed by confocal microscopy both as images and corresponding diagrams. **(A)** uninfected cells **(B)** *C. pneumoniae* MOI=1 for 6 hours **(C)** *C. pneumoniae* MOI=1 for 72 hours. Staining: chlamydial LPS (red), SOCS3 (green) and DNA (blue). Images were acquired and analysed with Leica confocal software. Data representative of 4 experiments. Original magnification 400x.

Figure 6.16

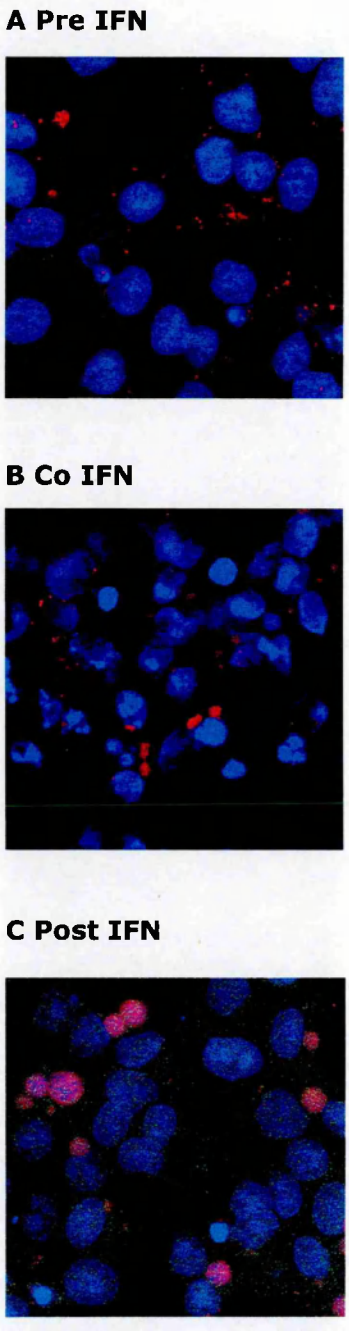


Figure 6.16 The effect of IFN-γ on SOCS3 expression in infected HEp-2 cells.**(A)** Pre-IFN **(B)** Co IFN **(C)** Post IFN. Cells were treated with 500 U/ml of IFN-γ and infected with MOI=1 of *C. pneumoniae*. Key: Pre- 24 hours prior to the infection; Co- at the time of the infection; Post- 24 hours after the infection. Staining: chlamydial LPS-red; SOCS3-green; DNA-blue. Data representative of 4 experiments. Original magnification 400x.

Figure 6.17

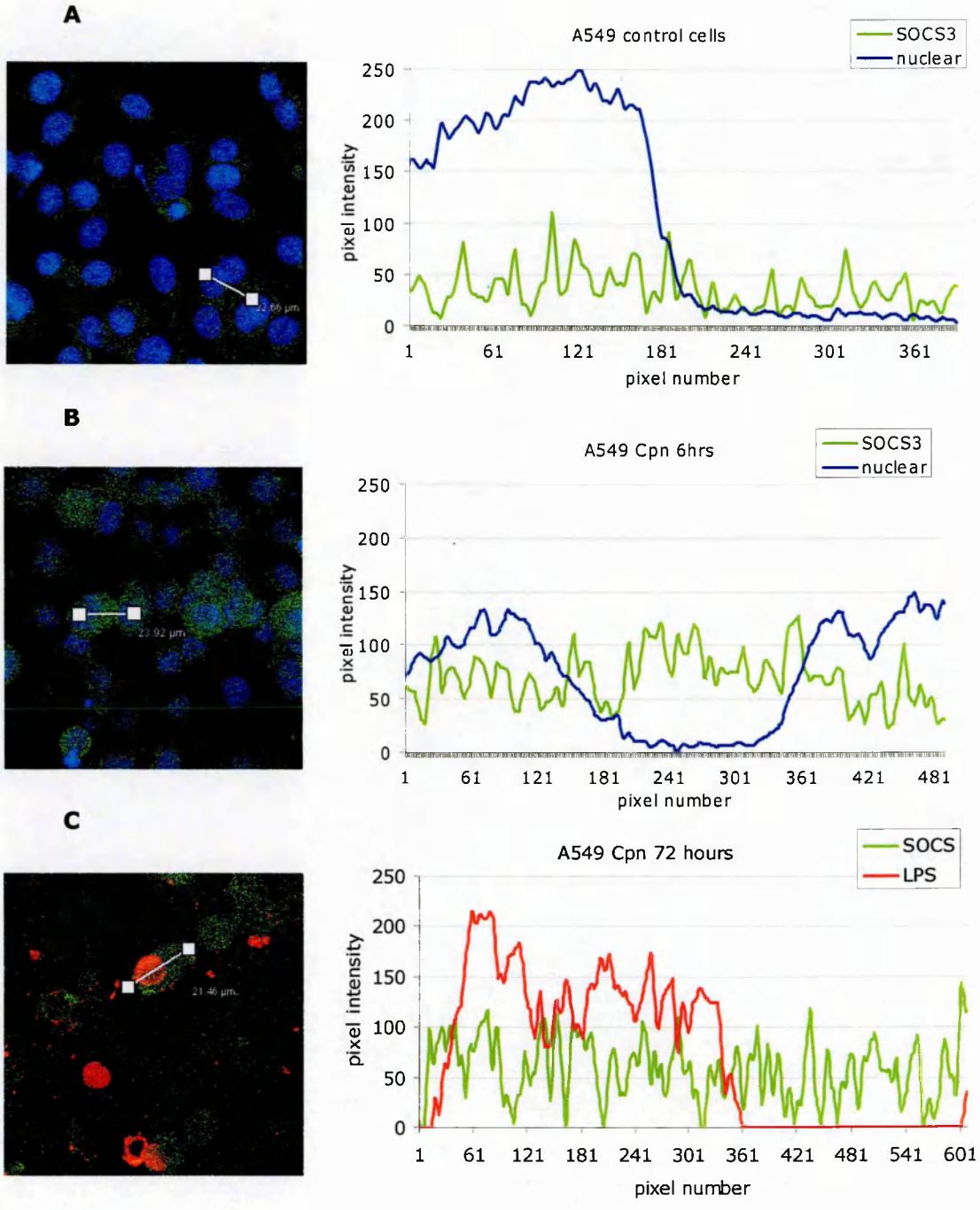


Figure 6.17 Expression of SOCS3 protein in A549 cells analysed by confocal microscopy both as images and corresponding diagrams. **(A)** uninfected cells **(B)** *C. pneumoniae* MOI=1 for 6 hours **(C)** *C. pneumoniae* MOI=1 for 72 hours. Staining: chlamydial LPS (red), SOCS3 (green) and DNA (blue). Images were acquired and analysed with Leica confocal software. Data representative of 4 experiments. Original magnification 400x.

Figure 6.18

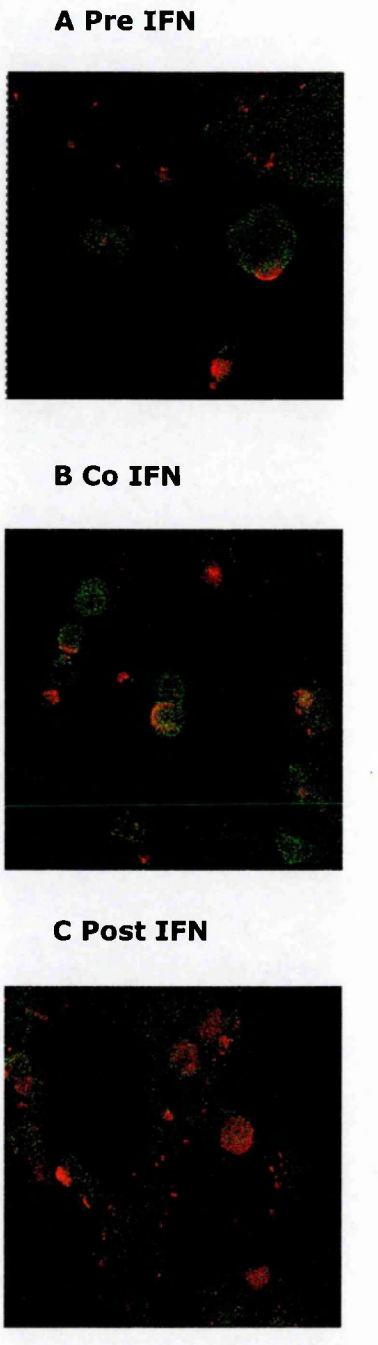


Figure 6.18 The effect of IFN-γ on SOCS3 expression in infected A549 cells.**(A)** Pre-IFN **(B)** Co IFN **(C)** Post IFN. Cells were treated with 500 U/ml of IFN-γ and infected with MOI=1 of *C. pneumoniae*. Pre- 24 hours prior to the infection; Co- at the time of the infection; Post- 24 hours after the infection. Staining: chlamydial LPS-red; SOCS3-green. Data representative of 4 experiments. Original magnification 400x.

In previous sections I discussed the role of LPS in the induction of SOCS3 expression. *S. minnesota* LPS did not induce SOCS3 mRNA or protein expression in either cell line. However *E. coli* LPS has previously been shown to induce SOCS3 expression. To confirm the results obtained earlier cells were treated with *S. minnesota* LPS and *E.coli* LPS as a positive control and analysed by confocal microscopy. *S. minnesota* LPS treatment for 6 hours does not induce SOCS3 expression in either cell line (Figure 6.19). On the other hand, *E. coli* LPS treatment up-regulates SOCS3 expression in A549 cells, and the staining intensity is comparable to chlamydial infection in both cell lines (Figure 6.20).

Figure 6.19

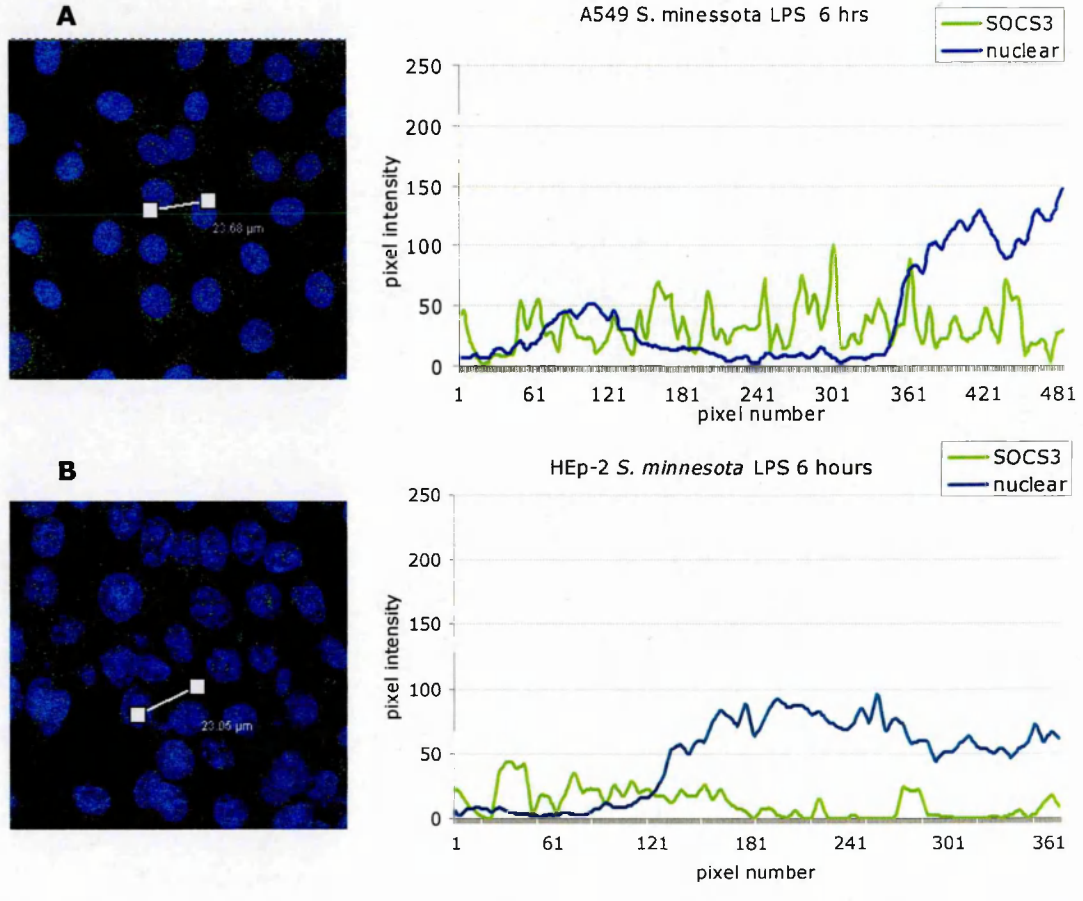


Figure 6.19 Effect of *S. minnesota* LPS treatment on SOCS3 protein expression both as images and corresponding diagrams. **(A)** A549 cells **(B)** HEp-2 cells. Cells were treated with 1µg/ml of LPS for 6 hours and then stained. Staining:SOCS3 (green); DNA (blue). Original magnification 400x.

Figure 6.20

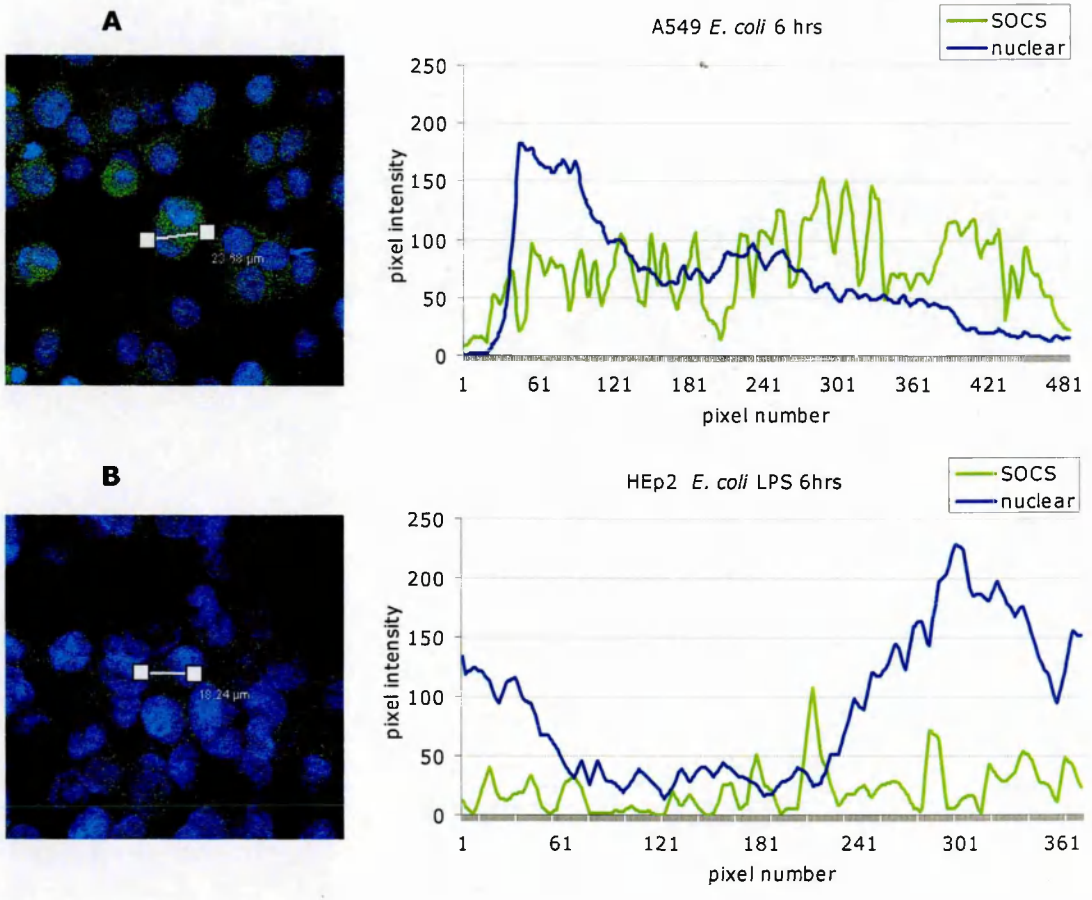


Figure 6.20 Effect of *E. coli* LPS treatment on SOCS3 protein expression both as images and corresponding diagrams. **(A)** A549 cells **(B)** HEp-2 cells. Cells were treated with 1µg/ml of LPS for 6 hours and then stained. Staining: SOCS3 (green); DNA (blue). Original magnification 400x.

One last attempt was made to investigate the expression of SOCS1 protein in response to *C. pneumoniae*, 72 hours after infection. In A549 cells there was background expression of SOCS1 and it was up-regulated by infection (Figure 6.21 A and B). HEp-2 cells did not show any expression of SOCS1 protein in resting cells, and 72 hours after infection there was low intensity staining (Figure 6.21 C and D).

Negative controls for all experiments discussed in this section were obtained by omitting the primary antibodies. There was no background in any samples (Figure 6.22).

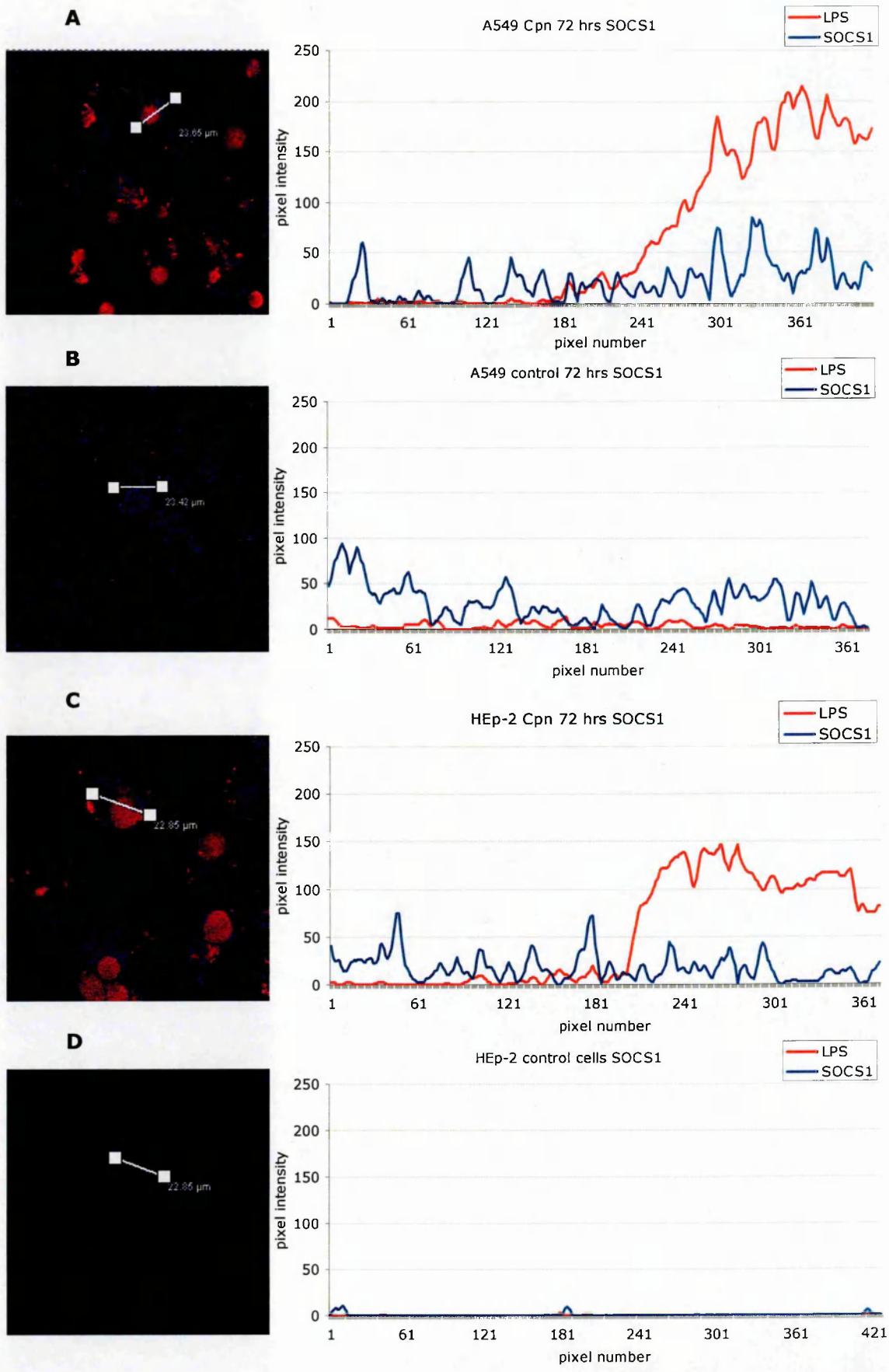


Figure 6.21 Expression of SOCS1 in A549 and HEp-2 cells. **(A)** infected A549 cells **(B)** uninfected A549 cells **(C)** infected HEp-2 cells **(D)** uninfected HEp-2 cells. Cells were infected with *C. pneumoniae* MOI=1 for 72 hours. Staining: SOCS1 (blue); chlamydial LPS (red). Original magnification 400x.

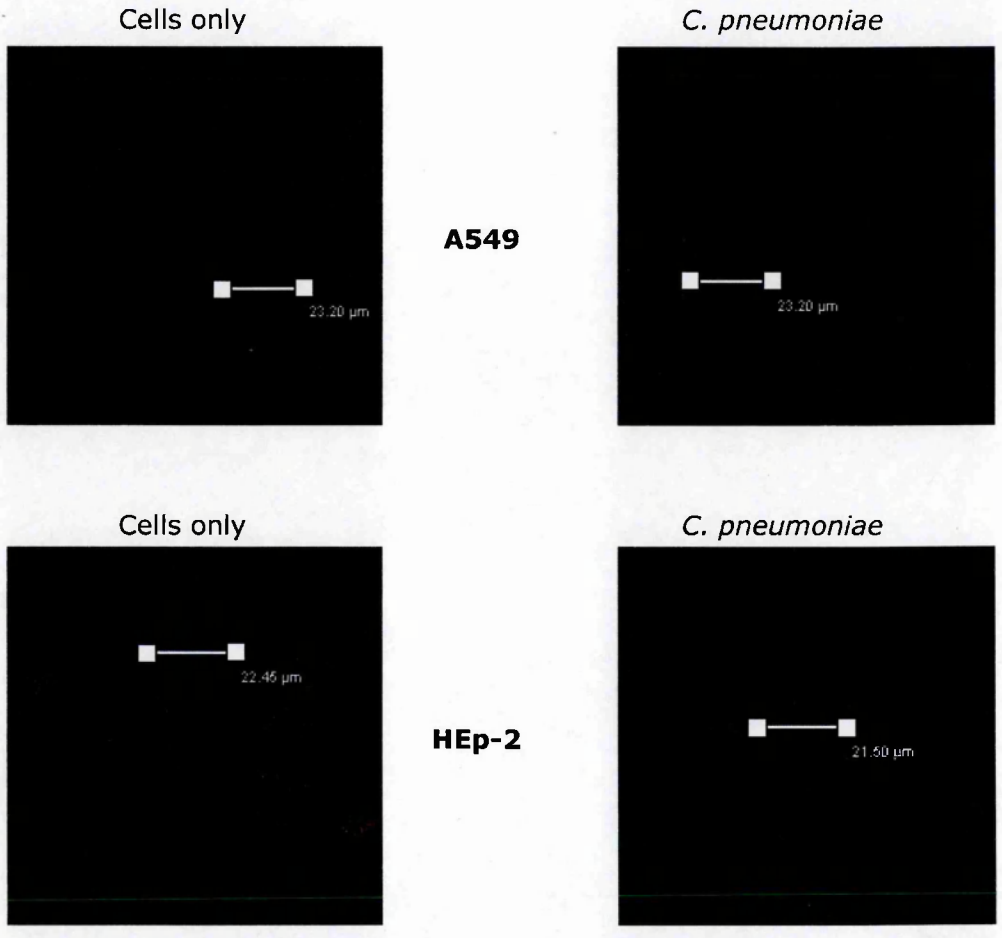


Figure 6.22 Negative controls for infected and uninfected cells. Negative controls were obtained by omitting the primary antibody. Original magnification 400x.

In summary, confocal microscopy proved to be the most suitable technique for the investigation of SOCS3 expression. After the initial optimisation the results were reproducible and the technique proved reliable. It also provided the largest amount of information about the patterns of SOCS expression. A549 cells express SOCS3 protein constitutively and HEp-2 cells do not. *C. pneumoniae* infection induces further protein expression in both cell types. When infected cells are treated with IFN-γ there is no change in A549 cells however there is suppression of SOCS3 expression in HEp-2 cells.

As an initial attempt to assign a function to constitutive SOCS3 expression in A549 cells, and considering the role of SOCS as inhibitors of Jak/STAT pathways, A549 cells were analysed for phosphorylation of STAT1 following IFN-γ treatment.

6.3.8 Stat1 phosphorylation in response to IFN-γ treatment in A549 cells

SOCS interfere with Jak/Stat signalling pathways and SOCS3 can inhibit activation of Stat1. IFN-γ signalling is largely Jak/Stat dependent, and to see whether constitutive SOCS3 expression in A549 cells is the reason behind their partial responsiveness to IFN-γ, phosphorylation of STAT1 was analysed.

A crucial step in activation of Stat1 is phosphorylation of tyrosine 701 (Y701). This was chosen for analysis in A549 cells following IFN-γ treatment. Cells were treated with 500 U/ml of IFN-γ for 5, 10 and 30 minutes and then analysed by western blotting. A549 cells show constitutive STAT1 Y701 phosphorylation and this is not changed by IFN-γ treatment (Figure 6.23)

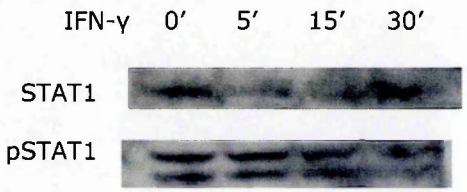


Figure 6.23 Tyrosine 701 STAT1 phosphorylation in A549 cells in response to IFN-γ treatment. Upper panel STAT1; lower panel phosphorylated STAT1 (pSTAT1).

6.4 *Discussion*

Early production of IFN-γ following *C. pneumoniae* infection has been to shown to be a crucial factor in host immune control and protection against disease (Rottenberg et al., 1999, Rottenberg et al., 2000, Rothfuchs et al., 2004). Lung epithelium is the first target for *C. pneumoniae* infection and is a site of multiplication of the organism. Therefore, any inability of these cells to respond to IFN-γ is likely to be detrimental to the host.

In this chapter two lung epithelial cell lines are described (HEp-2 and A549) with differential responsiveness to IFN-γ and differential ability to control the growth of *C. pneumoniae*: HEp-2 cells controlled the growth as shown previously, while A549 cells did not (Figure 6.2). Both HEp-2 and A549 cells express IFN-γ receptor and are

responsive to IFN-γ insofar as they express IDO mRNA following treatment (Table 6.1 and Figures 6.4 and 6.5). The failure of A549 cells to control *C. pneumoniae* is therefore surprising since tryptophan degradation by IDO has been shown to be the major mechanism of chlamydial control by IFN-γ in human cells *in vitro* (Beatty et al., 1994), and failure of IFN-γ to induce IDO results in uncontrolled chlamydial growth (Entrican et al., 2002). Since IFN-γ-mediated control of chlamydial growth is highly dose-dependent, this finding suggested that IDO message was not translating into control of *C. pneumoniae*, either through lack of functional protein or qualitative differences in IFN-γ signalling in A549 cells. This prompted investigation of SOCS expression.

Both A549 and HEp-2 cells express SOCS3 mRNA in the resting state and chlamydial infection induces up-regulation of SOCS3 expression at both mRNA and protein level (Figures 6.7 and 6.15 to 6.18).

During this investigation I encountered a number of difficulties in finding the best method in for detection of SOCS3 protein expression. Western blotting proved inconsistent and unreliable. The levels of protein in resting cells varied from blot to blot and no consistent trends could be seen (Figure 6.10). Immunohistochemistry proved to be better with more consistent results (Figure 6.11 and 6.12). However, the pattern of SOCS3 staining in infected cells was very similar to that of chlamydial inclusions thereby making the results inconclusive. Nevertheless, when the primary antibody was omitted there was no staining at all and the results were comparable to control samples (not shown). The question remained whether the staining was 'true' and whether SOCS3 protein localises in the vicinity of chlamydial inclusions in the host cell. In addressing these issues confocal microscopy proved to be the most sensitive and the most suitable technique.

SOCS3 protein is cytoplasmic and its location seems to change as the inclusion develops. Early after infection the protein is found diffusely in the cytoplasm, whereas

it becomes associated with the inclusion as it matures (Figure 6.17 B and C) and in the later stages it is in the vicinity of the inclusions (Figure 6.18). This raises the question that SOCS3 might be associating with chlamydial components in the inclusion membrane. One potential candidate is the Inc family of proteins that are putatively secreted into the host cell cytoplasm from the inclusion and thereby can interfere with a variety of host cell pathways (Rockey et al., 1997, Wyrick, 2000). Secretion of these proteins may be a way by which *C. pneumoniae* communicates with the host cell and how it can manipulate the host-cell responses.

In an effort to identify the chlamydial component(s) responsible for the up-regulation of SOCS3 following infection, the cells were treated with *Salmonella minnesota* LPS, which is similar to chlamydial LPS in both structure and endotoxic activity. It was found that it did not change SOCS3 expression; however *E. coli* LPS treatment did (Figure 6.19 and 6.20). There are differences in the structure and endotoxic activity between chlamydial and *E. coli* LPS, and this may influence their differential ability to induce SOCS3. This finding is in keeping with other reports suggesting that chlamydial LPS although antigenic, is not a potent inducer of inflammatory responses, and that it is the secretion of cHSP 60 that induces inflammation (Kol et al., 1998, Kol et al., 1999, Kol et al., 2000). Other bacterial components such as CpG, and other intracellular pathogens, including *Listeria monocytogenes* and *Mycobacterium tuberculosis,* can induce SOCS1 and SOCS3 expression, and this correlates with inhibition of IFN-γ signalling and the ability of the host cells to control the infection (Dalpke et al., 2001, Imai et al., 2003, Stoiber et al., 1999, Stoiber et al., 2001).

In investigating how IFN-γ modulates SOCS3 expression, I showed that IFN-γ treatment enhanced SOCS3 mRNA expression in infected HEp-2 cells but not in infected A549 cells (Figure 6.7). In addition, IFN-γ alone induces SOCS3 mRNA expression in uninfected HEp-2 cells but has a limited effect on uninfected A549 cells. It is interesting to note that Song and Shuai have previously shown that over-expression of SOCS1 and SOCS3 in epithelial cells inhibits control of viral infections by

type I and type II interferons (Song and Shuai, 1998), thus putting these findings together with the fact that A549 cells express SOCS3 protein constitutively may explain their partial responsiveness to IFN-γ treatment. The effect of IFN-γ on SOCS3 protein expression was only analysed in relation to *C. pneumoniae* infection and the pattern of SOCS3 expression followed that of chlamydial control. In HEp-2 cells, IFN-γ treatment prior to infection inhibited SOCS3 expression, while in A549 cells there was no change. In retrospect it would have been interesting to analyse also the effect on SOCS3 protein expression by IFN-γ alone by confocal microscopy, in order to get a more complete picture of how SOCS3 expression is controlled.

Induction of SOCS3 expression following *C. pneumoniae* infection in the lung epithelium is potentially beneficial to the pathogen as it prevents the development of protective Th1 type immunity and favours Th2 type responses, that have been shown to be detrimental to the host (Rottenberg et al., 1999, Rottenberg et al., 2000). Cells infected by *C. pneumoniae* are induced to release different inflammatory mediators and they also release chlamydial components. These may include LPS, Inc and hsp60, all of which may modulate IFN-γ signalling. Infected epithelial cells produce IL-6 and PGE_2 following chlamydial infection, and these mediators both induce SOCS3 expression (Jahn et al., 2000, Gasperini et al., 2002). SOCS3 expression controls the range of responses to IL-6, as demonstrated by selective deletion of *socs3* gene. The lack of SOCS3 causes a wider transcriptional response to IL-6 that is dominated by IFN-γ-regulated genes, owing to an excess of STAT1 phosphorylation (Croker et al., 2003, Lang et al., 2003). PGE_2 down-regulates Th1 type T cell responses (reviewed by (Harris et al., 2002) and there is some indication that SOCS3 is preferentially expressed in Th2 CD4+ T cells, and that it is associated with the inhibition of Th1 type responses and the release of pro-inflammatory cytokines (Seki et al., 2003, Egwuagu et al., 2002). Some reports have demonstrated high expression of SOCS3 in Th1 CD4+ T cells, nevertheless still with an inhibition of Th1 type responses (Anderson et al., 2003). Given that Th1 type immunity, and IFN-γ in particular, is important for protection from chlamydial infections, our findings propose a novel way by which *C.*

pneumoniae could manipulate the immune response in the early stages of the infection, thereby contributing to the establishment of persistence, that can lead to serious chronic conditions such as atherosclerosis and coronary heart disease.

As discussed earlier, SOCS interfere with JAK/STAT signalling pathways and IFN-γ signalling is largely Jak1/Jak2/STAT1 dependent. SOCS3 has an effect on IFN-γ signalling through inhibition of STAT1 phosphorylation (Stoiber et al., 1999). Binding of IFN-γ to the receptor causes STAT1 phosphorylation on tyrosine 701 (Y701) via Jak kinases. To act with maximal efficiency as a transcription factor, STAT1 must also be phosphorylated on serine 727 (S727) and this happens through the p38 kinase pathway (Ramana et al., 2002, Goh et al., 1999). Given the constitutive expression of SOCS3 in A549 cells it could be assumed that STAT1 phosphorylation is inhibited. However these cells show constitutive STAT1 Y701 phosphorylation and this is not changed by IFN-γ treatment.

Considering these opposing results, there are a few possible explanations. SOCS3 might be preventing secondary phosphorylation of STAT1 at S727, and there are also other inhibitors of cytokine signalling that could be involved. Protein Inhibitors of Activated STATs (PIAS) are a novel group of inhibitory molecules that inhibit the translocation of already activated (phosphorylated) Stats thereby preventing the Jak/STAT dependent signals (Wormald and Hilton, 2004). PIAS1 inhibits STAT1 and hence IFN-γ signalling. For PIAS 1 to inhibit it, STAT1 needs to be phosphorylated on Y701 (Liu et al., 1998, Ungureanu et al., 2003). Also, high constitutive expression can cause lack of responsiveness to IFN-γ treatment (Coccia et al., 2002).

Taking these findings into consideration it is clear that my investigation of IFN-γ unresponsiveness could have been expanded to include analysis of PIAS1 expression and, instead of taking phosphorylation of STAT1 as a marker of activation, I could have investigated translocation of STAT1 into the nucleus by confocal microscopy.

Nevertheless I believe that the experiments conducted show a potentially important role of SOCS proteins in chlamydial infections.

In summary, the results presented in this chapter demonstrate a complex, intimate host-pathogen interaction whereby *C. pneumoniae* may induce biochemical changes in the host cells that are favourable to its long term survival. Induction of SOCS3 expression may be one of the many pathways induced in the early stages of the infection and diminish IFN-γ-dependent control mechanism, thereby contributing to the establishment of persistent infection.

CHAPTER 7

PRELIMINARY INVESTIGATION INTO THE ROLE OF ELAFIN AND SLPI OVEREXPRESSION IN CONTROLLING CHLAMYDIAL GROWTH

7.1 *Introduction*

The presence of small cationic antimicrobial proteins in rabbit and guinea pig granulocytes was first reported in the mid-1960s (Zeya and Spitznagel, 1966a, Zeya and Spitznagel, 1966b, Zeya and Spitznagel, 1966c) and they have recently emerged as an important factor in the innate immunity of plants and animals alike. Research in recent years suggests that antimicrobial peptides are a key component of the innate immune responses of the lung. There are a number of different antimicrobial peptides; however a substantial portion of studies investigating the role of these peptides has focused on defensins (Lehrer et al., 1993, Kagan et al., 1994, McCray, Jr. and Bentley, 1997, Singh et al., 1998, Lehrer and Ganz, 2002).

7.1.1 Defensins

Defensins are a large family of antimicrobial peptides, contributing to the antimicrobial action of granulocytes, and mucosal host defence in the small intestine, in the lung and elsewhere. Structurally they can be divided in three groups: α-, β- and θ-defensins. α-defensins are small peptides with approximately 30 residues, cysteine rich, mostly expressed by granulocytes and some lymphocytes (Selsted et al., 1985); β-defensins are slightly larger (approximately 45 residues), with different cysteine pairing, and they are expressed by epithelial cells in skin and lung (McCray, Jr. and Bentley, 1997, Harder et al., 1997); θ-defensins were recently found in leukocytes of rhesus monkeys, are smaller and have a circular structure (Tang et al., 1999).

Defensins have a broad spectrum of activity against Gram-positive and Gram-negative bacteria (Ganz et al., 1985, Lehrer et al., 1989, Shafer et al., 1988, Miyasaki et al., 1991, Miyasaki et al., 1990, Ogata et al., 1992, Kohashi et al., 1992) as well as

against fungi (Lehrer et al., 1986, Lehrer et al., 1988) and enveloped viruses (Daher et al., 1986, Nakashima et al., 1993, Welling et al., 1998, Yasin et al., 2000, Bastian and Schafer, 2001, Quinones-Mateu et al., 2003). Defensins show synergistic activity with other host defence molecules such as lysozyme and lactoferrin (Singh et al., 2000). The microbicidal activity of defensins stems from their ability to permeabilize anionic lipid bilayers, release cellular contents and destroy the membrane's electrical potential.

Expression of α-defensins is increased in the airway secretions of patients with inflammatory lung diseases such as cystic fibrosis, COPD, chronic bronchitis and acute respiratory distress syndrome. Their role as effectors of the immune response in the lung seems to be two fold; they direct antimicrobial effects and cause immunomodulation that could contribute to immunopathology (reviewed in (van Wetering et al., 1999, Lehrer and Ganz, 2002). They have chemotactic properties, recruit inflammatory cells, activate T cells and epithelial cells; decrease levels of glutathione (a powerful anti-oxidant) in epithelial cells which can lead to oxidative damage, and by binding protease inhibitors released by the epithelium α-defensins can promote neutrophil-induced damage (Lillard et al., 1999, van Wetering et al., 1997, Yang et al., 1999, Yang et al., 2000). α-defensins also contribute to exacerbations of COPD by increasing bacterial adherence to the epithelial cells (Gorter et al., 1998).

There is evidence that defensins have bactericidal activity against intracellular bacteria such as *Mycobacterium tuberculosis* (Sharma et al., 1999, Sharma et al., 2000, Fu, 2003). Also α-defensins and protegrins were shown to have bactericidal effects on *C. trachomatis* growth in McCoy cells (Yasin et al., 1996b, Yasin et al., 1996a) There have been no reports on the effects of antimicrobial peptides on *C. pneumoniae*; however given the importance of defensins in the chronic diseases of the lung they may have a role in *C. pneumoniae* infections.

7.1.2 Proteinase Inhibitors

Proteinase inhibitors (PIs) are important molecules involved in the acute-phase response (APR), an important part of the initiation and development of inflammation. PIs are produced either locally in the mucosal sites, or systemically in the liver. Secretory leucocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ELAFIN) are two PIs produced locally at the site of injury and have very low concentrations in the circulation. Other PIs such as α_1-antichymotrypsin and α_1-protein inhibitor (α1-PI) are produced in abundance by the liver with a high concentration in the circulation (Perlmutter et al., 1989).

Expression of SLPI and ELAFIN is up-regulated by inflammatory signals, including cytokines such as IL-1 and TNF-α, LPS, neutrophil elastase and defensins (Sallenave et al., 1994, Jin et al., 1998), while anti-inflammatory cytokines such as TGF-β down-regulate their expression (Jaumann et al., 2000). The function of SLPI and ELAFIN seems to be anti-inflammatory, as they can inhibit the expression of TNF-α and matrix metalloproteinases (Jin et al., 1998, Zhang et al., 1997). Antiproteinases have also been shown to have "defensin"-like antimicrobial activities. They have antimicrobial properties *in vitro* against bacteria, fungi and, potentially HIV (Hiemstra et al., 1996, Simpson et al., 1999). Simpson *et al* have also shown by adenovirus-mediated gene transfer over-expression, that ELAFIN is active against *Pseudomonas aeruginosa* infection in the mouse lungs *in vivo* (Simpson et al., 2001). SLPI and ELAFIN were first isolated from the sputum secretions from patients with COPD as the most abundant elastase inhibitors (Hochstrasser et al., 1981, Kramps and Klasen, 1985, Sallenave and Ryle, 1991). They are also up-regulated in a number of other pulmonary diseases, including cystic fibrosis, acute respiratory distress syndrome, pneumonia and possibly asthma (reviewed in (Sallenave, 2000). Their role in these conditions is still under investigation, but it seems that they may be relevant in the onset of inflammatory responses, and they are also interesting as potential therapeutic targets.

Given their importance in lung pathology I decided to investigate the role of ELAFIN and SLPI in *C. pneumoniae* infections by studying how over-expression of these genes influenced the rate of chlamydial growth in lung epithelial cells.

7.2 *Experimental Approach*

Adenovirus-mediated over-expression of ELAFIN and SLPI was optimised by Sallenave et al (Sallenave et al., 1998), and the experiments described here were conducted using the same system. Briefly, A549 cells were incubated at 37°C in IMDM supplemented with 5% FCS (IMDM + 5%) and grown to confluence on 8 well chamber slides. Cells were gently washed twice with PBS and then incubated for 1 hour with one of the following treatments; Ad-ELAFIN in IMDM+5%; Ad-SLPI in IMDM+5%; empty virus Ad-DL70 in IMDM+5%; or IMDM+5% only with no virus added. Adenovirus was applied at a multiplicity of infection (MOI) of 100 PFU/cell (particle forming units). After 1 hour the cells were washed with PBS twice and fresh IMDM+5% was added, before incubation for 48 hours. Cells were then washed with PBS and infected with *C. pneumoniae* at MOI of 20, 2 or 0.2 inclusion forming units (IFU), cultured for another 72 hours, fixed and stained for chlamydial inclusions as described in section 2.6.2.1 of chapter 2. The results were analysed by confocal microscopy (Leica TCSNT confocal system; Leica Microsystems Heidelberg GmbH, Germany). Evaluation was done by counting the number of inclusions in a field representative of the sample at the original magnification of 200x. The rate of protection was calculated as the percentage of the total number of inclusions in untransfected cells.

7.3 *Results and Discussion*

Before presenting the results, it is important to mention that there were problems with the experimental design: the results showed that the transfection with the control viral construct (Ad-DL70) induced more cell death compared to ELAFIN and SLPI. It seems that this "non-replicating" construct had picked up the necessary genes for replication and was no loner of use as a control. This was confirmed after the experiments presented in this chapter were finished. However, a brief summary of the results and ideas for further experiments are presented in the following section.

The summary of the results is presented in Table 7.1 as number of inclusions per cells in a field. Over-expression of ELAFIN partially restricted chlamydial growth in A549 cells. At MOI=20 of *C. pneumoniae* the rate of protection is about 20% and there is a similar level of protection when cells are treated with MOI=0.2. At MOI=2 the transfection with ELAFIN seems to enhance the susceptibility of the cells to *C. pneumoniae* infection. Similar results are seen with SLPI transfection where cells are more susceptible (by ~100%) than un-transfected cells, while there is some protection at *C. pneumoniae* MOI=0.2.

Table 7.1 Number of inclusions per/cell in response to different adenoviral treatment. N/A= not applicable.

ADENOVIRALT REATMENT MOI=100	***C. PNEUMONIAE* MOI** 20	2	0.2
UNTRANSFECTED CELLS	**0.302**	**0.032**	**0.017**
AD-ELAFIN	**0.265**	**0.057**	**0.012**
AD-SLPI	**0.629**	**0.081**	**0.011**
EMPTY VIRUS AD-DL70	**N/A**	**N/A**	**N/A**

As can be seen in Figure 7.1, when the transfection of A549 cells with Ad-ELAFIN was followed by a high dose of *C. pneumoniae* (MOI=20), the cells looked unhealthy and appeared to be dying, with some cells detaching from the slide. Fewer cells were visible in the field compared to un-transfected cells, and there were some signs of apoptosis (condensed and fragmented nuclei; Figure 7.2 A; Ad-ELAFIN). Lower doses

of *C. pneumoniae* did not have such a detrimental effect on the cells (Figure 7.1 B and C; Ad-ELAFIN).

SLPI transfection did not damage the cells to the same extent but also failed to inhibit chlamydial growth (Figure 7.1 A to C Ad-SLPI). SLPI-transfected cells were much healthier in culture, and their numbers were much higher.

The detrimental effects of Ad-ELAFIN transfection combined with a high dose of *C. pneumoniae* suggest that it might be the condition of the host cells that is limiting chlamydial growth.

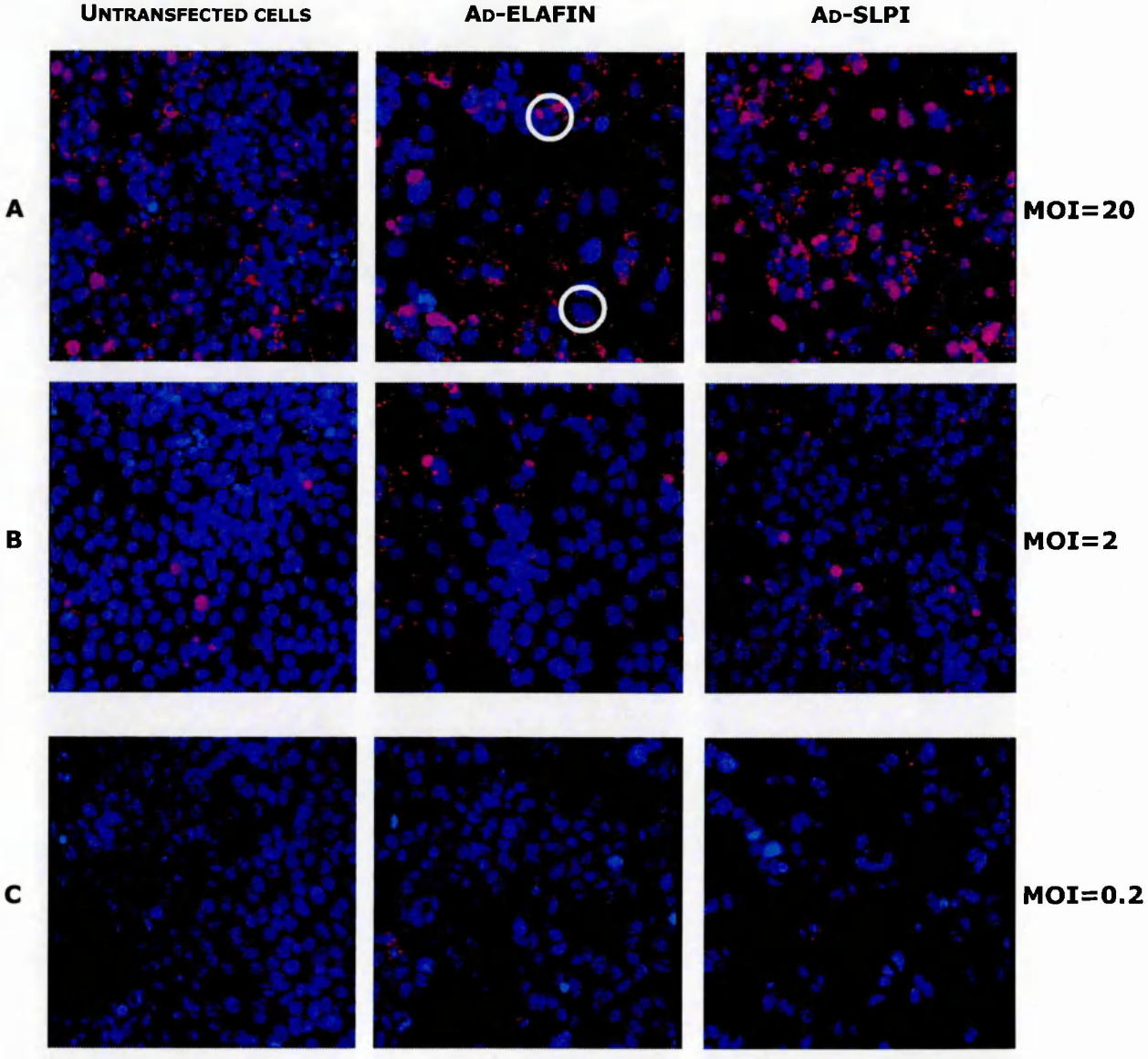


Figure 7.1 Effect of ELAFIN and SLPI over-expression on chlamydial growth. **(A)** *C. pneumoniae* MOI=20 **(B)** *C. pneumoniae* MOI=2 **(C)** *C. pneumoniae* MOI=0.2. A549 cells were transfected with MOI=100 of Ad-ELAFIN or Ad-SLPI and 48 hours later infected with *C. pneumoniae.* After 72 hours the cells were fixed and stained with α-chlamydial LPS to visualise chlamydial inclusions (red) and the nuclei were counterstained with To-pro 3 (blue). Original magnification 200x. White circles – apoptotic nuclei.

As discussed at the beginning of this section, it was established that the control virus has entered a replicating cycle. This may explain why transfected cells looked very unhealthy within 24 hours of chlamydial infection irrespective of the chlamydial dose, and after 72 hours most of the cells had detached and were dead. In comparison, both ELAFIN- and SLPI- transfected cells appeared healthier in culture. Since most of the cells were dead at the point of collection, it was difficult to fix and stain them. When they were stained, it was clear that *C. pneumoniae* is capable of developing inclusions in these cells; however it was impossible to count their number due to the clumping of the cells (Figure 7.2).

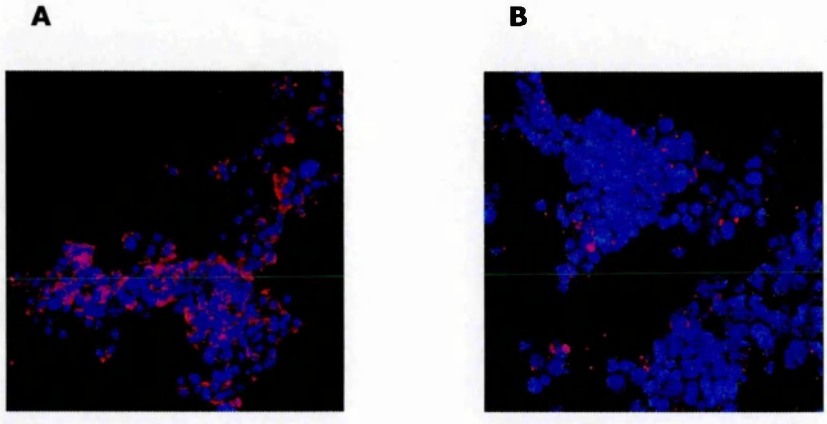


Figure 7.2 Chlamydial growth in cells transfected with the control virus. **(A)** *C. pneumoniae* MOI=20 **(B)** *C. pneumoniae* MOI=2. A549 cells were transfected with MOI=100 of Ad-DL70 control construct, and 48 hours later they were infected with *C. pneumoniae*. After 72 hours the cells were fixed and stained with α-chlamydial LPS to visualise chlamydial inclusions (red) and the nuclei were counterstained with To-pro 3 (blue). Original magnification 200x.

The fact that the control transfection caused more cell death compared to ELAFIN and SLPI transfection suggests that ELAFIN and SLPI do convey at least some protection from the detrimental effects of adenoviral transfection, and potentially *C. pneumoniae*. To summarise, transfection of cells with ELAFIN seems to induce a degree of protection against *C. pneumoniae* infection and a number of other experiments were planned, but due to the failure of the experimental design they were not done. Nevertheless, I outline these experiments:

- Titre out the adenovirus to a minimum dose that still gives a good expression of the target gene using an Ad-GFP construct and evaluate by fluorescence microscopy.

- Optimise the length of incubation with the virus before chlamydial infection.
- Since it is clear that *C. pneumoniae* MOI=20 is too high a dose, titre down the Chlamydia and use a dose that does not damage the cells as much. Also lower doses would be more similar to a real infection in the lung.

Once the experimental approach was optimised, the investigation could be expanded to include other read out systems. To assess the effects of ELAFIN and SLPI on the rate of chlamydial growth, determine the relative number of inclusions per number of cells. Also analyse the expression of chosen chlamydial genes at mRNA level by Real time RT-PCR. To determine the effect of SLPI and ELAFIN on Chlamydia-induced inflammation, investigate secretion of cytokines by the transfected cells compared to un-transfected cells.

The experiments outlined here, would, I believe, give an rounded picture of the role of ELAFIN and SLPI in chlamydial infections in the lung. Given the importance of antiproteinases in chronic lung diseases such as COPD and cystic fibrosis, and given the association of *C. pneumoniae* with these diseases, it is highly likely that ELAFIN and SLPI contribute to building protective immunity against *C. pneumoniae* and also to the outcome of the infection.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Atherosclerosis and coronary heart disease are fast becoming the number one cause of death in the developed countries. Saikku et al., (1988) were the first to report the presence of *Chlamydia spp.* TWAR (*Chlamydia pneumoniae*) in atherosclerotic plaques and suggested a correlation between chronic infection and atherosclerosis. Since then numerous investigations and clinical studies have been conducted to confirm the association of *C. pneumoniae* infection and the development of atherosclerosis, however this still remains an area of controversy.

Lung epithelium is the primary target of *C. pneumoniae* infections. To better understand how *C. pneumoniae* may contribute to the development of chronic conditions, early immune responses and host-pathogen interactions in the lung need to be dissected and understood. The work described in this thesis was designed to investigate a number of early responses in human infections with a view to better understand how early immune responses might contribute to persistence. It was shown that host cells are activated to release cytokines following infection, and that the cytokine profile depends on the cell type (presented in Chapter 4); that *C. pneumoniae* modulates the expression of cell-surface molecules in MdMs and that infected epithelial cells activate MdMs (presented in Chapter 5); and that *C. pneumoniae* induces inhibitory intracellular pathways (SOCS3) in lung epithelial cells that may interfere with the control of chlamydial growth in the host cell (presented in Chapter 6). A preliminary study into the role of natural antimicrobials in controlling *C. pneumoniae* growth was conducted and it indicated that these molecules may contribute to control and clearance of *C. pneumoniae* (presented in Chapter 7).

Events following *C. pneumoniae* infection are a combination of pro- and anti-inflammatory responses and I will try to summarise them here. Infected epithelial cells and MdMs both produce IL-8 and IL-6, while MdMs also release IL-1β and TNF-α. The

amount of IL-8 and IL-6 released by infected epithelial cells is increased by IL-17 treatment. Infection of MdMs increases the levels of CD14, CD40 and CD54 surface expression. All of these responses promote inflammation by recruitment and activation of neutrophils (IL-8), activation of Th1 type responses (IL-1β, TNF-α, IL-6, up-regulation of CD40 and CD54) and suppression of CD4+CD25+ regulatory T cells (IL-6) and also increase the cells' susceptibility to *C. pneumoniae* infection (up-regulation of CD14). These responses taken together may contribute to chronic inflammation and the development of atherosclerosis. However the pro-inflammatory responses are balanced by the activation of regulatory/inhibitory events. Infected MdMs produce IL-10, but no IL-12, while the amount of IL-8, TNF-α and IL-1β released by infected MdMs shows a downward trend when treated with IL-17. Levels of MHC class I and II molecules on MdMs decrease following infection, while CD45 levels increase. *C. pneumoniae* also induces the expression of SOCS3 in lung epithelial cells. The anti-inflammatory responses can dampen the inflammation and promote persistent infection in a number of ways: inhibiting the control of chlamydial growth through lack of IL-12 production thereby reducing the amount of IFN-γ; inducing expression of SOCS3 and up-regulation of CD45 that may both interfere with IFN-γ mediated control; and promoting immune evasion through down-regulation of MHC class I and II, thereby inhibiting antigen presentation and processing, and activation of adaptive immune responses.

In addition to host cells responding to infection, they can also activate other cells, thereby modulating the cells' ability to participate in immune responses. It was shown that infected epithelial cells activate MdMs by modulating the expression of cell-surface molecules. Unlike the infection, CM-treatment of MdMs down-regulates CD14 and CD45 expression, while up-regulating MHC class I and II and also CD91 expression. This kind of response may prime the MdMs to be less sensitive to *C. pneumoniae* infection and more efficient in controlling chlamydial growth (down-regulation of CD14 and CD45), while becoming more efficient in activation of adaptive immune responses (up-regulation of MHC class I and II and CD91).

A schematic of the early immune responses analysed in this project is presented in Figure 8.1.

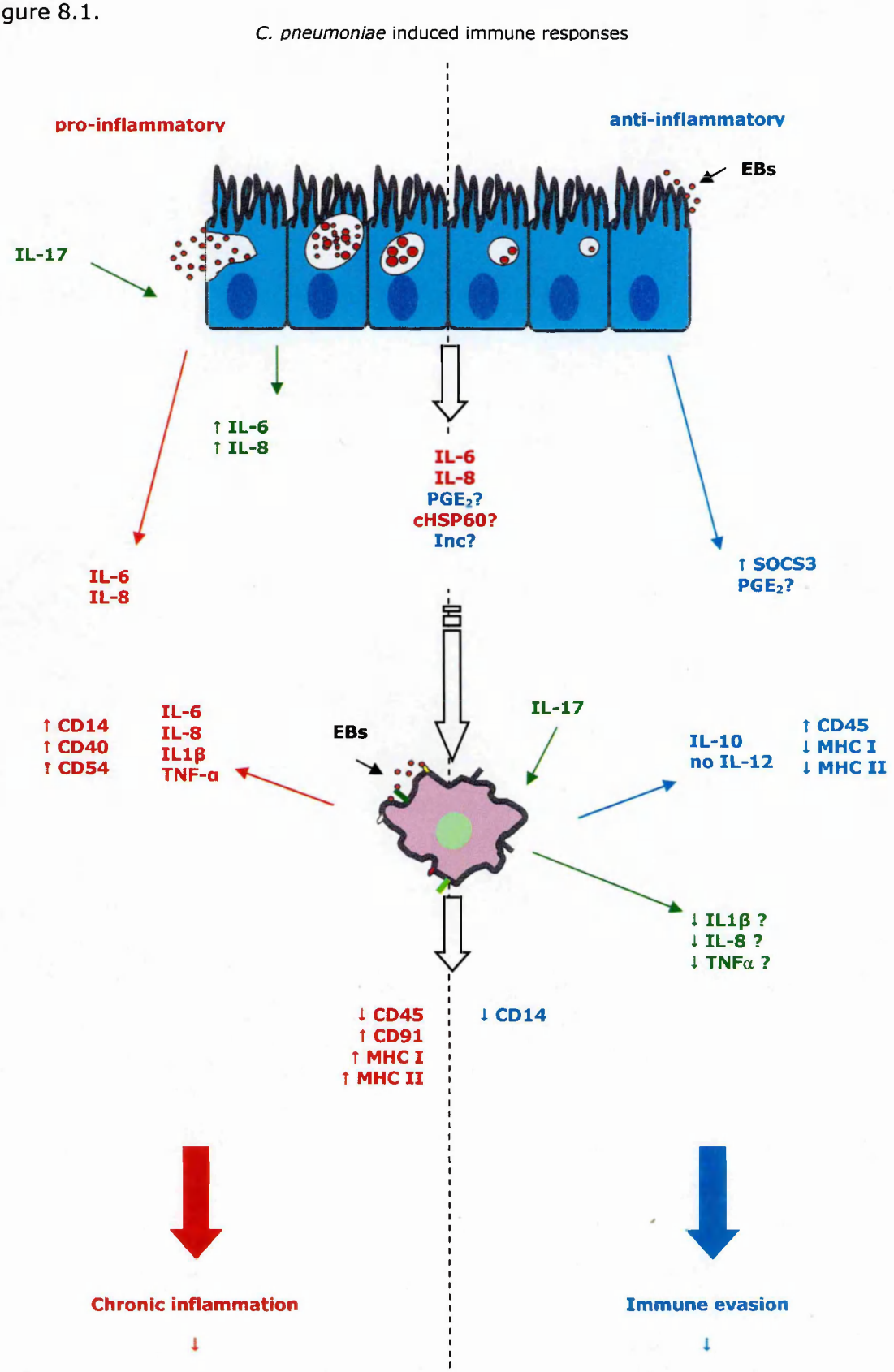


Figure 8.1 Immune responses analysed in this project. Responses are divided in pro- and anti-inflammatory. Key: **RED LETTERS AND ARROWS** - pro-inflammatory response; **BLUE LETTERS AND ARROWS** – anti-inflammatory response; **GREEN LETTERS AND ARROWS** – effects of IL-17 treatment of infected cells; **WHITE ARROWS**- CM treatment. **EBs** - elementary bodies.

As I discussed before, it is difficult to dissect out the immune responses in such a way as to determine the sequence of events following the infection, because of the number of contributing factors. From the data presented and discussed in this thesis and previous research, it is becoming clear that the early immune responses may play a crucial role in the final outcome of *C. pneumoniae* infection. There is a low-level inflammatory response that is beneficial for the long-term survival of the pathogen. A strong inflammatory response would cause eradication of the organism, while a lack of inflammation might allow the bacterium to multiply very quickly, leading to the death of the host. Long-term, persistent infections with a low-level inflammatory response may contribute to the development of chronic diseases such as atherosclerosis and COPD that *C. pneumoniae* is associated with.

At the beginning of this project an *in vitro* model was established and described (presented in Chapter 3) in order to analyse early immune response to *C. pneumoniae* infection in human cells. Investigating immune responses in humans is very difficult for a number of reasons. The reagents for analysis are abundant; however the experiments are almost exclusively done *in vitro*, unless they are a part of a clinical study, and even then the responses may be measured *ex vivo*. With *C. pneumoniae* there is the added problem of the high seroprevalence in the population, meaning that obtaining samples from naïve individuals is almost impossible.

In the current model a combination of established human lung epithelial cell lines and primary blood-derived MdMs was used. Using cell lines is technically simple and the samples are abundant, however they are mostly derived from cancerous tissue and are genetically different both from primary tissue and from each other. The two cell lines used in this project are of different origins; HEp-2 cells are derived from a larynx carcinoma and A549 cells from a lung carcinoma. There are differences in the way they respond to *C. pneumoniae* infection. They show similar kinetics of chlamydial growth; however they differ in the amount of LPS released from infected cells when infected with the same MOI. They preferentially release different cytokines following infection: A549 release more IL-8 than IL-6, while HEp-2 release more IL-6 than IL-8.

The major difference between A549 and HEp-2 cells is their ability to control chlamydial growth in response to IFN-γ. This phenomenon was a very interesting basis for investigating the role of SOCS in chlamydial infections.

Another issue arising from working with human models is the analysis that can be conducted. The investigations are largely observational, with limited manipulation of responses possible such as treatment with blocking antibodies, transfection of cells with a particular gene (eg. adenoviral gene therapy) or gene silencing (RNA interference; RNAi). In future studies it would be ideal to combine the *in vitro* human work with mouse models where the observations may be taken further and there is more scope for manipulation of the immune responses.

As a result of the research conducted in this project there are a few interesting avenues that may be pursued in future.

- Detailed analysis of the role of IL-17 in *C. pneumoniae* infections *in vivo*.

IL-17 modulates cytokine production by infected cells *in vitro* (Chapter 4). In future investigations it would be interesting to analyse the presence of IL-17 in the lungs of infected mice. For that purpose bronchial lavage fluid (BAL) may be used for both Real time RT PCR and protein analysis (Western blots rather than ELISA). To determine the role of IL-17 in the lung infections, different avenues could be pursued by manipulation of IL-17 expression: over-expression of IL-17 receptor, blocking IL-17 function with a blocking antibody or using IL-17 knock out mice in order to block the effects of IL-17; over-expression of IL-17 through gene transfer for the analysis of the opposite effects. A number of different responses could be measured, such as cytokine production, the composition of the cell population in the lung (BAL), bacterial load and infection clearance. Similar experiments were conducted by Ye et al (2001a and 2001b) in the investigation of the role of IL-17 in *K. pneumoniae* infections.

- The role of CD91 in *C. pneumoniae* infections

Given the importance of HSPs in chlamydial infection, it would be interesting to expand the investigation into the role of CD91. In the current study it was shown that CM-treatment of MdMs up-regulates CD91 expression, while infection had a mixed effect. Considering the size of the sample analysed (n=4 for infection, n=3 for CM-treatment), this should first be expanded to confirm current results. Further analysis might include blocking CD91 and monitoring any differences in the production of cytokines and other markers of MdM activation such as CD80/86 and MHC class I and II molecules; investigating by confocal microscopy the localisation of the receptor following infection and also analysing the contents of CM to confirm that it contains cHSPs and/or human HSPs. The investigation could be expanded *in vivo* in a similar way to the experiments proposed for IL-17 to get an idea whether CD91 has a crucial role in anti-chlamydial immune responses.

- How does the expression of cytokine signalling inhibitors (SOCS, PIAS, CD45) affect the outcome of *C. pneumoniae* infections?

The current investigation has demonstrated that infected epithelial cells express SOCS3. There was also an indication that over-expression of SOCS3 (as in A549 cells) impairs the cell's ability to control chlamydial growth in response to IFN-γ treatment. Stat1 phosphorylation was also analysed in A549 cells, however it was shown that it is phosphorylated (p-Stat1) constitutively. To confirm that Stat1-dependent signalling is impaired it would be interesting to analyse the localisation of p-Stat1 by confocal microscopy instead of by western blotting. This would confirm the level at which IFN-γ signalling is inhibited in A549 cells. To expand this, other inhibitors such as PIAS1 could be analysed by Real time RT-PCR and confocal microscopy. Other *in vitro* experiments could include silencing of *socs1* and *socs3* genes with RNAi or over-expression by gene transfer, and in both cases subsequent analysis of *C. pneumoniae* growth rates in epithelial cells.

In vivo analysis into the role of SOCS3 is difficult since SOCS3 knock out mice do not survive beyond the embryonic stage. In a seminal paper into the role of SOCS3 in IL-6 signalling Croker et al (2003) used Cre-loxP site-specific recombination to knock the

socs3 gene from target tissues (liver and macrophages). In future studies this technique could be used for targeted deletion of the *socs3* gene in the lung, and analysis of the impact on cytokine profile, bacterial load and the outcome of infection.

Before this project started, there was already a heated scientific discussion on the role of *C. pneumoniae* infections in the development of atherosclerosis and other chronic diseases. The results presented in this thesis support the idea that *C. pneumoniae* infection in the lung may contribute, or even cause the development of chronic diseases such as atherosclerosis and COPD through inducing a low-level inflammatory response. However a lot more research is needed to understand the underlying mechanisms of host - C. *pneumoniae* interactions, and design novel therapies and safe and effective prevention strategies.

9 APPENDICES

9.1 Appendix 1-Solutions

9.1.1 PBS (MRI Scientific Services)

900 ml dH_2O

8.0 g NaCl (Sigma Aldrich)

1.15 g Na_2HPO_4 (Sigma Aldrich)

0.2 g KH_2PO_4 (Sigma Aldrich)

0.2 g KCl (Sigma Aldrich)

Adjusted to 1 L in volume at pH 7.4, autoclaved and stored at RT

9.1.2 Trypsin/Versene (MRI Scientific Services)

20% Trypsin solution (MRI Scientific Services)

40.0 g NaCl (Sigma Aldrich)

3.8 g KCl (Sigma Aldrich)

0.5 g Na_2HPO_4 (Sigma Aldrich)

15.0 g Tris (hydroxymethyl)methylamine (Sigma Aldrich)

7.5 ml 1% Phenol red solution (Sigma Aldrich)

12.5 g Trypsin (Difco Laboratories, Surrey, UK)

Made up to 5 L in deionised water pH 7.6

80%Versene solution (MRI Scientific Services)

48.0 g NaCl (Sigma Aldrich)

1.2 g KCl (Sigma Aldrich)

6.9 g Na_2HPO_4 (Sigma Aldrich)

1.2 g Versene (EDTA; Ethylene Diamine Tetra acetic acid (Disodium salt: Dihydrate)) (Sigma Aldrich)

9.0 ml 1% Phenol red solution (Sigma Aldrich)

Made up to 6 L in deionised water pH 7.2

9.1.3 Chlamydial Transport Medium (MRI Scientific Services)

373 g Sucrose (Sigma Aldrich)

2.560 g KH_2PO_4 (Sigma Aldrich)

6.185 g K_2HPO_4 (Sigma Aldrich)

3.605 L-glutamic acid (Sigma Aldrich)

Made up to 5 L in deionsed water

20 ml 0.4% Phenol Red solution (Sigma Aldrich) added

500 ml FCS (GIBCO BRL) added

25 ml (1/4 vial) Gentamycin (GIBCO BRL) added

0.5 g Streptamycin (GIBCO BRL) added

Filtered and 25 ml 10000 U/ml Nystatin suspension added

9.1.4 Red Cell Lysis Buffer

79 mg NH_4HCO_3 (1mM)

6 g NH_4Cl (114mM)

Made up to 1 L and filter sterilised

9.1.5 Agarose gels for RNA or DNA

9.1.5.1 RNA gels

Add 0.8g agarose (Promega) to 80 ml of 1x MOPS/EDTA buffer and dissolve it in a microwave at low heat.

Mix well, cool under tap to ~60°C and add 4ml of 37% formaldehyde (Sigma-Aldrich). Pour into tray with comb in place and let it set. Place tray in the tank filled with 1x MOPS.

Mix 2µl of RNA sample with 8µl of loading buffer and leave at 65°C for 10 minutes. Cool instantly on ice.

Load samples and run at 100V for 30-45 minutes. Visualise under UV light.

10x MOPS ([3-(N-morpholino) propanesulphonic acid]) /EDTA buffer

41.86 g MOPS (0.2M) (Sigma Aldrich)

16.7 ml 3M Sodium acetate (0.05M, pH 4.8)

20.0 ml 0.5M EDTA (0.01M)

Add to 800ml of dH_2O, dissolve and adjust pH to 7.0 and make up to 1L.

Use 1:10 to make up working solution.

RNA loading buffer

0.75 ml Formamide

0.15 ml 10x MOPS

0.24 ml Formaldehyde

0.1 ml Glycerol

0.1 ml 10% Bromophenol blue

0.01 ml 10mg/ml Ethidium bromide

0.1 ml dH_2O

9.1.5.2 DNA gels

Add 0.8g-1.6g (1-2%) of agarose in 80 ml of 1xTBE and dissolve it in a microwave at low heat.

Mix well, cool under tap to ~60°C, pour into the tray and let it set.
Transfer the tray in the tank filled with 1x TBE containing 5µg/ml of ethidium bromide.
Mix 5µl of DNA with 1µl of 6x DNA loading buffer (Promega) and load the samples.
Run the gel at 80-100V for 45-90 minutes as needed. Visualise under UV light.

10X TBE

108g Tris
55g Boric Acid
40ml 0.5M EDTA

Add to 800 ml of dH_2O, mix well and make up to 1 L. To make a working solution dilute 1:10 and add 5µg/ml of ethidium bromide.

Ethidium Bromide solution

Add 0.1 g of ethidium bromide to 10ml dH_2O mix on magnetic stirrer for several hours. Wrap in foil and store at RT.

9.1.6 Detachment Buffer

12mM Lidocaine-HCl (Sigma-Aldrich)
10mM EDTA (From 0.5M pH 8 stock)
in PBS (see 11.1.1)

9.1.7 Protein Lysis Buffer

50 mM HEPES pH=7.5 (stock 0.5 M; 1:10)	11.9g	
50mM NaCl (stock 5 M; 1:100)	2.92g	
50mM NaF	2.1g	in 1 L of dH_2O
10mM Na pyrophosphate	4.46g	

0.5 M Na_3VO_4 (stock 10mM; 1:20)

Add protease inhibitor cocktail (Complete R, Roche Diagnostics ltd, Lewes, UK) 1 tablet per 15 ml of lysis buffer

9.1.8 Western Blot Buffers

MOPS buffer (see 9.1.5.1)

Transfer Buffer

3.03g Tris
14.4g Glycine
800ml dH_2O
200ml Methanol

Stripping Buffer

0.75 g Tris
2 g SDS (Sodium dodecyl sulphate; Sigma-Aldrich)

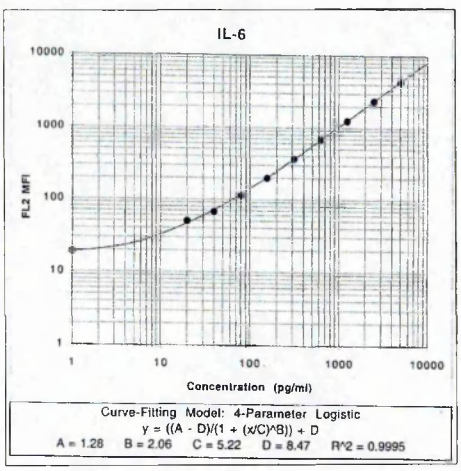
Add to 100 ml of dH_2O. Prior to use add 700µl of β-mercaptoethanol (Sigma-Aldrich). Cover membranes with buffer and leave at 50°C for 1 hr. Wash 3 times with wash buffer and re-stain membranes according to protocol.

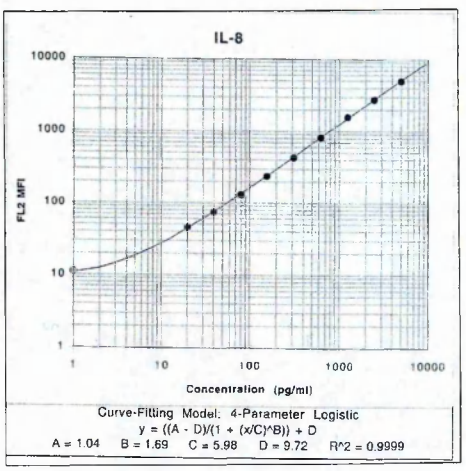
9.1.9 Mowiol mounting medium

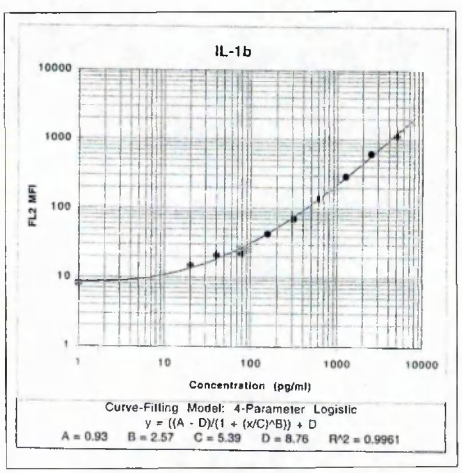
Add 2.4g Mowiol 4.88 (Sigma Aldrich) to 6g glycerol and stir briefly with a pipette
Add12ml dH_2O and stir at RT for several hours (best left overnight)
Add 12ml 0.2M Tris (pH 8.5) and heat to 50°C for 1-2hours while stirring
When the Mowiol has dissolved, clarify by centrifugation at 500x g for 15 mins
Add DABCO to 2.5% (0.72g), aliquot and store at –20°C. Bubbles can be removed by centrifugation. Aliquots can be stored for up to 2 weeks at 4°C.

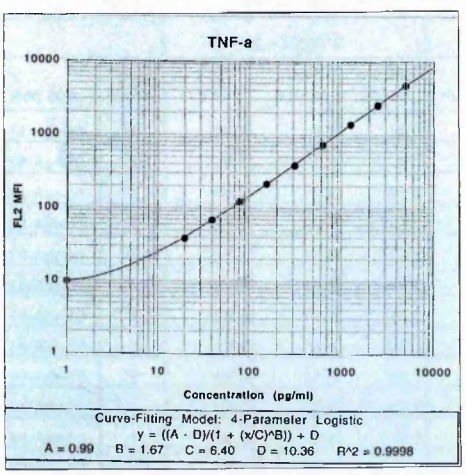
9.2 Appendix 2 – Standard Curves

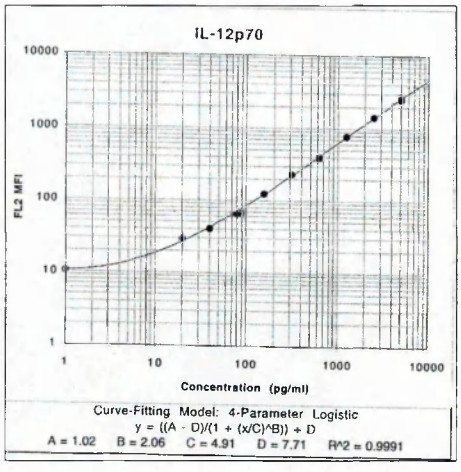
9.2.1 CBA standard curves

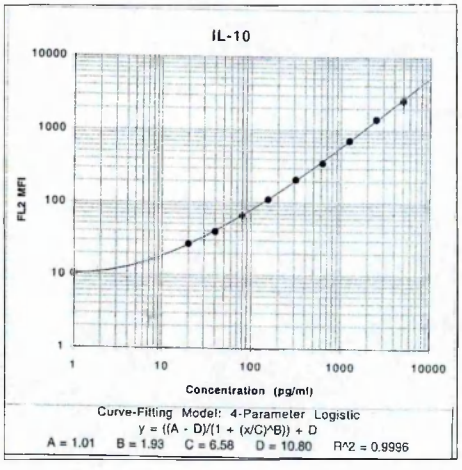






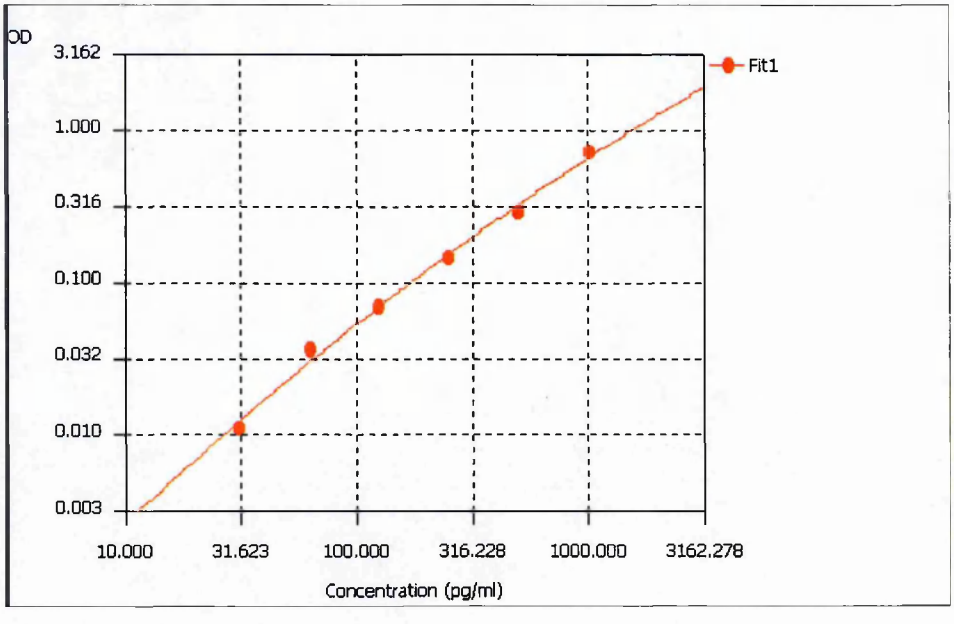






9.2.2 ELISA standard curves

9.2.2.1 IL-6



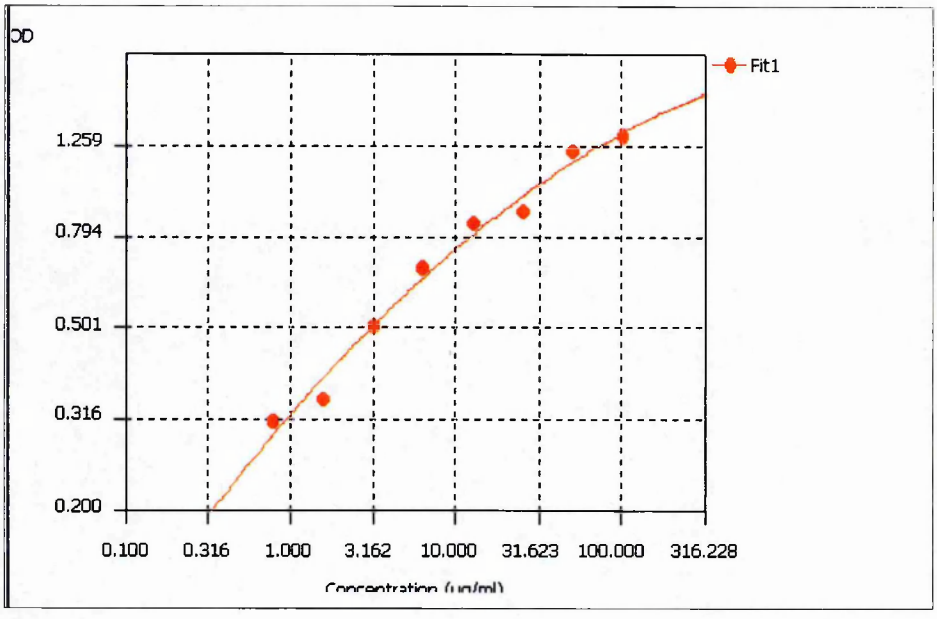
Log/log axes scaling

Quadratic regression with data extrapolation

$Y= -4.1007 +1.67307X -0.1070X^2$

R-Squared = 0.9930

9.2.2.2 LPS



Log/log axes scaling

Quadratic regression with data extrapolation

$Y= -4.867 +0.4193X -0.0552X^2$

R-Squared = 0.9848

9.3 Appendix 3- CBA raw data

The following tables present raw values of the CBA cytokine analysis in pg/ml. Some of the cells are in colour. Key: - values not available (samples lost or experiments not done); - values over the threshold of 5000 pg/ml; - values over the threshold even when diluted 10x.

9.3.1 HEp-2 cells

HEp-2 CELLS	IL-6	pg/ml				
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10 + IL-17**
d1	122.6	807.8	175.1	1299.9	280.9	958.1
d2	53.9	249	73.3	433.9	139.3	516.2
d3	77.8	361.4	93.6	927.7	140.1	648
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10 + IL-17**
d1	171.6	742.4	180.4	1405	566.3	2591.6
d2	77.4	431.6	91.7	563.4	442.8	1033.1
d3	82.5	378.3	94.6	543.4	234.4	1033.1
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10 + IL-17**
d1	621.6	1930.7	1257.5	**5000**	**5000**	**5000**
d2	272.6	1126.9	400	2143.9	**5000**	**5000**
d3	326.7	1243.7	456.5	2169.2	**5000**	**5000**

HEp-2 CELLS	IL-8	pg/ml				
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	4.6	24.5	12.3	51.8	40.2	16.1
d2	11.7	10.1	6.5	21.6	8.1	23.5
d3	8.7	14.5	6.5	35.9	10.6	17.1
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	11.2	29.2	13.1	88.2	29.5	220.7
d2	9.8	21.2	8.4	33.1	26.8	70
d3	12.1	21.8	11.6	23.5	15.8	77
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	29.2	137.3	78.6	458.5	**5000**	**5000**
d2	19.5	61	20.1	164.8	**5000**	**5000**
d3	17.4	63	25	166.5	**5000**	**5000**

9.3.2 A549 cells

A549	IL-6	pg/ml				
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	5.7	6.2	8.4	11.3	32.4	23.3
d2	9.4	5.7	5.6	8.5	33.6	40.6
d3	13.2	49.2	14.9		56	11.4
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	8	5.6	7.5	10.3	31.6	30.8
d2	1.6	4.5	6.2	9.8	31.2	28.5
d3	7.2	9.9	10.5	9.5	39.8	47
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1 +IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	4.2	10	14.2	10.2	72.1	38
d2	7.4	10.3	9.1	8.8	45.4	44.3
d3	9.8	14.5	13.1	16.2	63	52.7

A549	IL-8	pg/ml				
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	12.8	13.7	14.6	34.7	75.4	81.6
d2	14.1	18.1	20.1	32.4	88.7	187.9
d3	18.1	105.2	13.9		107.4	7.3
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	16.7	42	32.4	47.8	216.3	326.2
d2	27.5	51	35.9	124	349.9	589.5
d3	18	34.3	47.5	34.9	148.9	304.1
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL-17**
d1	721.5	1418.8	1502.3	1084.7	4518.1	3392.8
d2	566.2	1227.3	1279.1	2277.1	3999.7	**5000**
d3	560.6	1676.5	1062.7	1240.1	2561	2978

9.3.3 MdMs

IL-6				pg/ml		
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	9.3	3.1	5360	4729.4		
d2	11.2	9.35	9.1	10	7	9.5
d3	3.4	6.2	11060	6300	11290	9870
d4	8.9	6.6	12260	7490	12710	7740
d5	0	0	1230.1	1190.1	20570	27260
d6	2.6	0	5570	4640	82080	105580
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	6.7	8.8	3750	4232		
d2	5.8	11.4	18040	15500	21630	16150
d3	6.8	8560	7900	11770	11180	9970
d4	8.1	8.7	16810	11890	18230	8340
d5	0	0	958.1	1536.8	32670	38800
d6	32	10.4	5570		63470	87010
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	9.9	2.6	4540	4451		
d2	14.2	10.2	14010	14290	19760	22520
d3	2.1	6.8	22290	26980	12330	17680
d4	7.4	13.7	15430	5960	20990	6370
d5	11.4	0	989.5	994.5	56630	70450
d6	0	6.5	5630	6870	89840	170170

IL-8				pg/ml		
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	1086.2	1755	32290	25650		
d2	608.8	1089.5	569.2	983	439.6	896.6
d3	333.8	695.7	137530	87510	87510	83160
d4	314.8	410.2	118990	103050	122730	99930
d5	814.9	1020	43610	29510	256110	282140
d6	2047.7	2702.4	96910	83160		
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	2492.2	4159.7	35340	25650		
d2	390.5	1013.8	258870	27710	222920	194230
d3	428.8	88400	85740	176670	116570	118990
d4	520.3	901.2	209160	202620	202620	15740
d5	1020	1361.1	34990	57780	446820	467170
d6	3583.4	3583.4	116570			
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	7780	10630	50200	54940		
d2	1089.5	1653.5	285200	258870	301040	370320
d3	731.8	1159.1	328360	366280	175840	232620
d4	1779.6	1618.3	282140	171200	362290	184260
d5	2791.1	1921.9	40660	4940	1842.6	1942.3
d6	4619.9	4650	38960	146370		

IL-1β			pg/ml			
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	39.2	15	277.4	294.2		
d2	67.45	46.8	48.1	56.6	45.9	73.5
d3	9.1	7.9	24.9	61.1	356.6	379.4
d4	50.4	33	826.9	58.2	712.9	67.4
d5	0	0	0	0	351.3	445.8
d6	0	0	0	0	590.9	718.2
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	49.4	27.2	251.7	158.7		
d2	59.4	72.1	72.6	57.4	460.3	373.5
d3	274.2	32.3	29.6	0	288.6	
d4	33.4	59.9	908.1	56.6	1007.1	44.4
d5	0	0	0	0	1027.5	973.8
d6	0	0	0	0	1435.6	1660
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	62.2	9	258.7	187.1		
d2	67.4	47.4	0	0	156.1	118.1
d3	0	9.9	29.6	60	90.4	79.4
d4	31	58.6	633.2	41.5	666.5	73.9
d5	0	0	0	0	1700.6	1475.7
d6	0	0	0	0	2689.9	2268

IL-10			pg/ml			
24 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	15.8	5.7	22.8	36.2		
d2	13.05	10.5	7.9	7.8	11	15.5
d3	2.3	8.3	24.2	33.3	36.6	54.7
d4	12.8	9.5	336.8	178.6	348.3	204.7
d5	0	0	32.8	23	299.2	415.5
d6	0	0	49.5	37.7	654.5	575.7
48 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	7.7	10.1	30	21.1		
d2	11.4	14.4	71	64.2	81.7	83
d3	7.5	5.5	29.6	13.4	44.9	
d4	11.7	11.7	257.1	185.7	325.6	197.9
d5	0	0	27.7	33.8	454.2	635.4
d6	0	5.1	41.4	37.2	572.8	623
96 hours						
	control cells	**IL-17**	**MOI=1**	**MOI=1+IL-17**	**MOI=10**	**MOI=10+IL17**
d1	18.6	4.1	20.5	15.1		
d2	11.9	12.2	62.2	77.6	69.9	65.6
d3	1.9	5.9	31	35.7	30.3	26.8
d4	12.7	12.2	219.1	83.9	263.4	94.4
d5	3.5	0	29.3	19.8	359.8	467.8
d6	1.4	0	37.7	42.5	521.5	604.8

TNF-α			pg/ml			
24 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	5.4	2.2	648	784		
d2	6.8	5.95	4.7	5.7	4.7	5.4
d3	2.2	2.4	420	491.7	1321.7	1301.4
d4	5.5	4.9	1406.6	335.4	1363.5	313.3
d5	0	0	22.2	22.2	315.4	196.6
d6	0	0	55.8	42.3	300.6	546.8
48 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	7.8	9.2	189	210		
d2	5.5	7.7	48.9	50.6	587.6	682.5
d3	4.5	3.2	630	150.2	617.6	
d4	3.4	4	851.5	85	951.9	141.8
d5	0	0	0	0	55.8	50.8
d6	0	0	0	0	79.5	138
96 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	13.1	6.5	55.4	71.8		
d2	7.5	6.7	4.3	5.7	154.6	148.8
d3	2.2	4	52.4	94.5	208.5	208.5
d4	7.3	7.2	238.7	15.3	362.6	23.9
d5	0	0	0	0	33.2	28
d6	0	0	0	0	46.1	91.2

IL-12			pg/ml			
24 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	12.1	5.5	0	11.3		
d2	11.7	10.1	11.1	10.1	15.5	7.4
d3	2.9	7.9	8.3	13	7.8	9.2
d4	9.1	5.7	11.5	10.3	13.5	11.9
d5	0	1.2	1.3	0	0	0
d6	0	0	8.1	3.4	0	0
48 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	10.9	9.4	11.7	14.6		
d2	9.3	12.5	12.5	12	12.7	10.6
d3	5.5	6.2	7.6		6.9	0
d4	7.2	9.3	4.2	13.4	9.9	13.4
d5	1.6	0	2	0	0	3.2
d6	0	0	0	0	0	3
96 hours						
	control cells	IL-17	MOI=1	MOI=1+IL-17	MOI=10	MOI=10+IL17
d1	15.8	3.6	9.6	6.2		
d2	11.6	6.6	1.7	2.3	3.7	0
d3	3.6	3.1	7.2	11.7	5.8	2.7
d4	7.6	7.8	13.9	7.6	6.4	14.5
d5	1.2	1	0	2.1	0	1.3
d6	0	0	0	0	1.3	2.1

9.4 Analysis of cytokine data

Data for monocytes were available from six donors. Blood samples from each donor were each treated with six different reagents, and the presence of cytokines measured at three time points, 24, 48 and 96 hours respectively. For the first donor only four reagents were used (MO1=10 and 10+IL-17 were missing) giving a total of 102 measurements for the complete set.

Data for epithelial cells were available for 3 separate experiments for each cell line (HEp-2 and A549).

9.4.1 Statistical model

Because of the unbalanced nature of the data the REML directive was used in Genstat 7th edition to fit a model to the data. A repeated measures model was used with a uniform correlation structure (subjects were taken as the donor/treatment combinations or experiment/treatment combinations). This is equivalent to fitting a split-plot model with donors designated as blocks, treatments as main plots and time as split plots. The data for each cytokine were positively skewed and so all analyses have been carried out using log transformed data, $\log_{10}(x+1)$.

Below is an example (IL-10) of the analysis that was conducted on all experiments

IL-10 (log scale)

Table of raw means in log scale

TIMES/HR	24	48	96
TREATMENTS			
MOI=1+IL-17	1.545	1.619	1.590
MOI=10+IL-17	2.131	2.445	2.140
IL-17	0.646	0.822	0.631
MOI=1	1.581	1.722	1.675
MOI=10	2.098	2.302	2.209
UT	0.672	0.678	0.839

***** **REML Variance Components Analysis** *****

Response Variate:	logIL-10
Fixed model:	Constant+Donors+Times+Treatments+Times.Treatments
Random model:	units.times
Number of units:	102

*** units.Times used as residual term with covariance structure as below**

* Sparse algorithm with AI optimisation

* Units with missing factor/covariate values included

* Units with missing data values included

***** Covariance structures defined for random model *****

Covariance structures defined within terms:

Term	*Factor*	*Model*	*Order*	*Nrows*
units.Times	units	Identity	1	34
	Times	Uniform	1	3

***** Residual variance model *****

Term	*Factor*	*Model(order)*	*Parameter*	*Estimate*	*S.e.*
units.Times			Sigma2	0.224	0.0563
	units	Identity	-	-	-
	Times	Uniform	theta1	0.7675	0.0691

***** Wald tests for fixed effects *****

Fixed term	**Wald statistic**	**d.f.**	**Wald/d.f.**	**Chi-sq prob**
*** Sequentially adding terms to fixed model**				
Donors	9.54	5	1.91	0.090
Times	4.90	2	2.45	0.086
Treatments	**63.88**	**5**	**12.78**	**<0.001**
Times.Treatments	5.03	10	0.50	0.889
*** Dropping individual terms from full fixed model**				
Times.Treatments	**5.03**	**10**	**0.50**	**0.889**
Donors	**7.53**	**5**	**1.51**	**0.184**

*** Message: chi-square distribution for Wald tests is an asymptotic approximation (i.e. for large samples) and underestimates the probabilities in other cases.**

***** Table of predicted means for Constant *****

1.515 Standard error: 0.0761

***** Table of predicted means for Donors *****

Donors	1	2	3	4	5	6
	1.543	1.433	1.274	1.913	1.397	1.529

Standard errors:	Average	0.1856
	Maximum	0.2247
	Minimum	0.1776

***** Table of predicted means for Times *****

Times/hr	24	48	96
	1.448	1.581	1.516

Standard errors:	Average	0.08274
	Maximum	0.08316
	Minimum	0.08253

***** Table of predicted means for Treatments *****

Treatments	MOI=1+IL-17	MOI=10+IL-17	IL-17	MOI=1	MOI=10	ut
	1.585	2.206	0.700	1.659	2.209	0.730

Standard errors:		
	Average	0.1848
	Maximum	0.1996
	Minimum	0.1776

***** Table of predicted means for Times.Treatments *****

Treatments	MOI=1+IL-17	MOI=10+IL-17	IL-17	MOI=1	MOI=10	ut
Times/hr						
24	1.545	2.137	0.646	1.581	2.104	0.672
48	1.619	2.336	0.822	1.722	2.308	0.678
96	1.590	2.146	0.631	1.675	2.214	0.839

Standard errors:		
	Average	0.2010
	Maximum	0.2239
	Minimum	0.1932

Conclusion:

There strong evidence that treatment of cells induces significant differences in IL-10 levels. There is no significant difference between donors and different times. There is a dose dependent response to *Chlamydia pneumoniae* infection, while IL-17 does not significantly affect the levels of IL-10 in either

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